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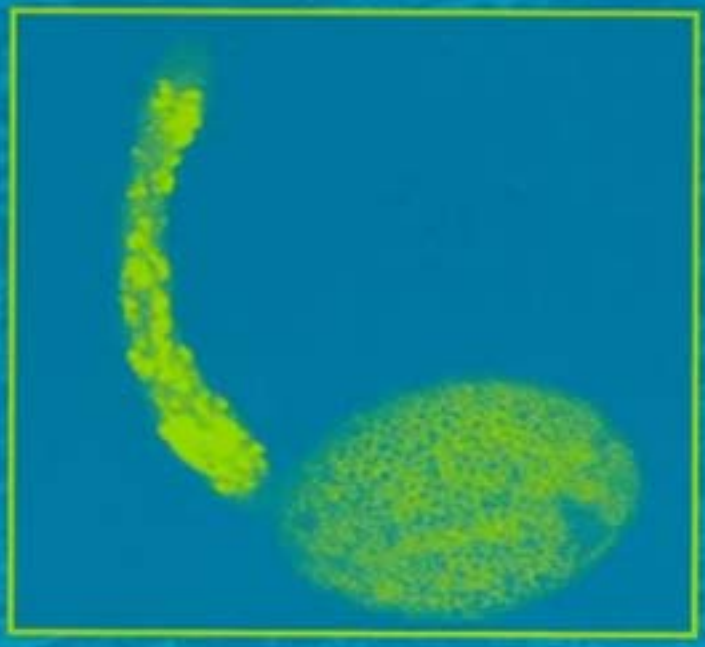

R. Malhó
(Ed.)

The Pollen Tube

A Cellular and Molecular Perspective

Springer

3
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Editor

R. Malhó is an associate professor in the Department of Plant Biology, Faculty of Sciences of Lisbon. He holds a Ph.D. in cell biology, with a major interest in signal transduction mechanisms and imaging of cell dynamics — in particular, the study of pollen tube growth and its signalling pathways. Dr. Malhó did post-doctoral work at the University of Edinburgh with A.J. Trewavas and has published 40 papers in international peer-reviewed journals and 8 book chapters.

Preface

In 1974 Springer published a book entitled *Pollen* by Stanley and Linskens. Years later, when I started researching, this was the first textbook on pollen and pollen tube growth I used. There I got acquainted with the ultrastructural aspects of pollen tubes, how they relate to cellular tip growth and to the progression in stigma and style until fertilization was achieved. The emphasis was then on structure and the most prominent questions at the time also reflected the available methodologies. It was therefore not surprising that studies on pollen tubes focused mainly on elucidating the role of the male gametophyte in double fertilization. Basic information gained on the different steps of fertilization is indeed a contribution to the promotion of genetic engineering in plants and crop improvement.

While this remains true, the outstanding technological advance registered in the past 30 years allowed us to change from a morphological/structural approach to a dynamic/functional approach. It is now evident that pollen tubes are excellent examples for plant cell research, particularly suitable for investigations on cell tip growth and polarization, signal transduction, channel and ion flux activity, gene expression, cytoskeleton and wall structure, membrane dynamics and even cell–cell communication.

The enormous amount of data about pollen tube growth presently available (about its physiology, biochemistry, cell biology, molecular genetics, etc.) clearly demonstrates the interest of the scientific community in this particular cell type and reflects its qualities as a biological model that go much beyond the carrier of the sperm cells essential for reproduction. The diversity of techniques and methodologies now used to study pollen and pollen tube growth is reflected in this book, which is written by biochemists, cell biologists, molecular biologists and geneticists, with obvious different perspectives but that, on the whole, see the cell as a complex network composed of structural and regulatory elements with an assembly and function modulated by internal and external stimuli.

The first chapter of the book introduces the general architecture of the pollen tube and how signalling pathways impinge on this structure. It follows with a chapter about the transcriptomic view of pollen development and how this methodology may help us pinpoint both structural and regulatory components of the pollen tube machinery. Among these are certainly ions, membrane

transporters, GTPases, phosphoinositides and signalling lipids. Together, these form an intricate signalling network modulating crucial processes (e.g. secretion and cytoskeleton dynamics) and are reviewed in the next four chapters. These are followed by chapters on the basic structural components of the pollen tube, but emphasize the dynamics and functional aspects of microfilaments, microtubules and the cell wall. The following two chapters review the interaction of the pollen tube with the female tissue and how this affects the male gametophyte at a molecular and cellular level. The mechanisms of self-incompatibility and guidance to the micropyle are key aspects of plant cell–cell communication that may elucidate us on the evolution of land plants. The last two chapters are conceptually complementary. First, an analysis and discussion of the potential of screening pollen tube mutations taking advantage of bioinformatic and genomic tools in order to identify key components of the pollen tube growth machinery. And naturally, the current model to perform such tests is *Arabidopsis thaliana*. However, in a few more years' time, with ongoing sequencing projects of several economically important species (among others), our choices will be significantly broader. The last chapter focuses precisely on the advantage of studying several species and the benefits we can expect from such diversity analysis.

As a final note, I would like to thank the authors for their work in producing such excellent chapters and the publisher for producing such a book. I also thank all those who contributed over the years to the extensive data now available. It was for me a real pleasure and honour to edit this fine book that, I hope, will serve as a valuable tool for scientists working in sexual reproduction and plants in general.

Lisbon, January 2006 R. Malhó

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The Pollen Tube: A Model System for Cell and Molecular Biology Studies

Rui Malhó

Faculdade de Ciências de Lisboa, ICAT, Universidade de Lisboa, 1749-016 Lisboa, Portugal
r.malho@fc.ul.pt

Abstract Pollen tubes, the active male gametophytes of seed plants, are the vectors carrying the male sperm cells to the egg cell of the female gametophyte in the ovules of seed plants. Unlike most plant cells in which growth occurs by modification of the existing wall and the insertion of new material throughout its surface, pollen tubes extend strictly at their apex, undergoing a specialized type of growth called tip growth. Consequently, these cells exhibit a highly asymmetric functional behaviour in processes such as ion fluxes, secretion, wall assembly and cytoskeletal arrangements. This spatial segregation is very attractive for cell biology studies. But the pollen tube can also be regarded as a single haploid cell carrying the sperm cells and thus of great interest for genetical and molecular studies. Last, but not least, pollen is easy to germinate under in vitro conditions, where tubes can grow extremely rapid, making it accessible to application of a wide range of technologies. Therefore, it stands as an ideal system for cell and molecular studies. Here I review some of the basic concepts of pollen tube growth (which are thoroughly discussed in subsequent chapters), address current paradigms and how these are likely to be challenged by recent data that stress how dynamic these cells are.

1 Introduction

The pollen grain, upon germination on a receptive stigma, develops a pollen tube that grows through the pistil towards the ovule while carrying the sperm cells to the embryo sac. The two main functions of the pollen tube are then to elongate and to interpret the guidance cues from the female tissue. Despite this apparent simplicity and the large amount of data already available, many questions remain to be answered.

Pollen tubes are thought to derive from the haustoria by which the primitive microgametophytes fed on the host sporophyte. The initial steps of pollen germination consist of an extensive hydration process that will permit metabolism to resume. During this phase the volume of organelles increases proportionally (Malhó and Pais 1992). The entrance of water is likely to be driven by ion fluxes (Hepler et al., this volume), namely K^+ influx (Feijó et al. 1995) increasing the turgor pressure. A high turgor pressure will preferably stretch the plasma membrane at the germination pore of the grain because in this region an additional

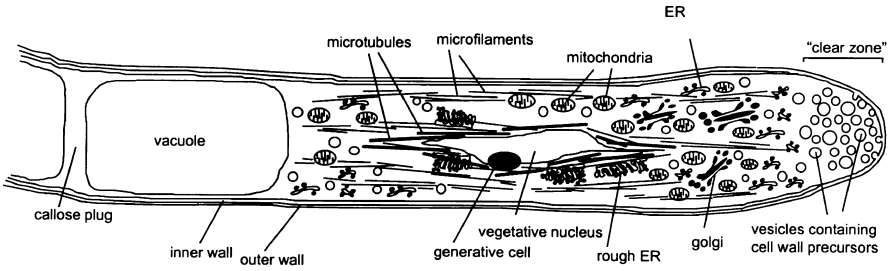

Fig. 1 Diagram depicting the intracellular organization of a pollen tube. The "four zones" classic zonation of a pollen tube is illustrated: apical or clear zone, sub-apical, nuclear and vacuolar. Reproduced from Franklin-Tong (1999). Copyrighted by the American Society of Plant Biologists. Reprinted with permission

exine wall is lacking. Stretch-activated channels (Sze et al., this volume) may open and cause a local depolarisation of the plasma membrane, a cation influx, and a local increase in $[Ca^{2+}]_c$ generating a positive feed-back mechanism.

The elongation of the emergent pollen tube is accomplished through a form of cell extension common in all eukaryotes from fungal hyphae to nerve cells: tip growth. This growth form serves as a paradigm for cell polarity because cell extension is restricted to a narrow zone at the apex. Although some differences exist between species, a general model for pollen tube ultrastructure considers four cytological domains (Cresti et al. 1977; Fig. 1): an apical or "clear zone", devoided or large organelles and packed with golgi vesicles that fuse with the apex delivering wall precursors (Malhó et al. 2005; Geitmann and Steer, this volume); a sub-apical region with a typical cytoskeletal arrangement (Yokota and Shimmen, this volume and Cai and Cresti, this volume) and rich in mitochondria, dictyosomes and endoplasmic reticulum; a nuclear zone where the vegetative nuclei and the sperm cells move; a vacuolar zone that enlarges as tube grows. The continued growth of the pollen tube causes the regular formation of cytoplasmic interruptions of callose ("callose plugs"; Heslop-Harrison, 1987). These plugs isolate the older vacuolated parts of the tubes and confine the cytoplasm to the front regions of the cell. This led to the suggestion, never properly confirmed, that the volume of cytoplasmic material remains constant over the whole process of growth until the mycropile (Malhó et al. 1992). Sanders and Lord (1992) took the implications of this suggestion a step further and proposed that pollen tubes should be viewed not as a growing cell but as a "moving" one (Johnson and Lord, this volume).

2
A Signaling Network Spatially Segregated

Signaling is an integral component in the establishment and maintenance of cellular identity. Tip-growing cells and pollen tubes, in particular, have often

been considered an ideal system to investigate signal transduction mechanisms partly because of the characteristics above mentioned. A direct proof that this is more than a nice sentence to put in introductions and abstracts is the fact that so many signalling pathways have been identified and a role assigned in germination and tip growth; ions (Ca^{2+}, H^+, K^+, Cl^-), calmodulin (CaM), phosphoinositides, phospholipids, protein kinases, cyclic nucleotides, 14-3-3 proteins and GTPases (Malhó and Camacho 2004; Hepler et al., this volume–Zárský et al., this volume). These constitute a large and complex web of signaling networks that intersect at different levels (Fig. 2) such as the con-

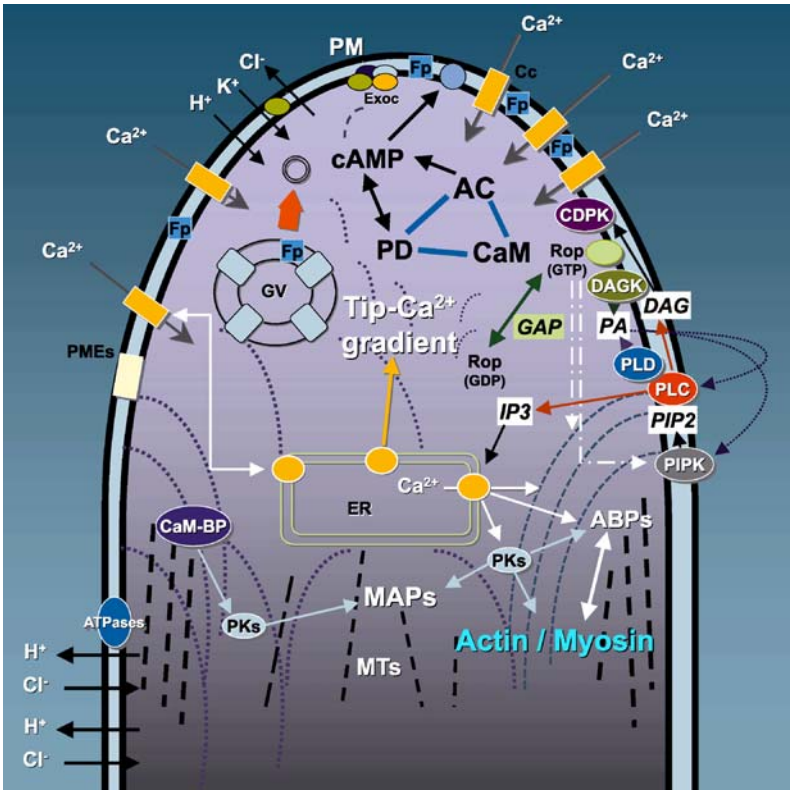

Fig. 2 Apical region of a growing pollen tube illustrating the main signalling transduction pathways and their components. A close interaction between the different pathways foresees the existence of a highly complex, signalling loop in this region, capable to interpret simultaneous (and possibly contradictory) extracellular cues. ABPs, actin-binding proteins; AC, Adenylyl cyclase; CaM-BP, Calmodulin-binding protein; Cc, Ca^{2+} channels; DAG, diacylglycerol; DAGK, DAG kinase; Exoc, Exocyst; Fp, Fusogenic protein (SNAREs and/or anexins); GAP, Rop GTPase activating protein; GV, Golgi vesicle; IP3, Inositol 1,4,5 triphosphate; PD, Phosphodiesterase; PIP2, phosphatidylinositol-(4,5)-bis phosphate; PIPK, phosphatidylinositol kinase; PLC, phospholipase C; PLD, phospholipase D; PM, plasma membrane; PMEs, pectin-methyl-esterases; R, IP3 receptor

trol of vesicle targeting/fusion, the physical state of the actin cytoskeleton, cell wall assembly and extracellular communication (Yokota and Shimmen, this volume–Johnson and Lord, this volume).

2.1 Ca^{2+}, Central Regulator or Follower?

Cytosolic free calcium ($[Ca^{2+}]_c$) is a key element in the regulation of pollen tube elongation and guidance. A tip focused $[Ca^{2+}]_c$ gradient has been imaged with a high 1–3 μM Ca^{2+} concentration in the tip region and a low 0.2–0.3 μM Ca^{2+} concentration in the subapical and basal part of the tube (see detailed discussion in Hepler et al., this volume). Disruption of this gradient leads to inhibition of tube growth and available data indicates its involvement in the control of cytoplasmic streaming (Zárský et al., this volume, and Yokota and Shimmen, this volume), cell wall assembly (Geitmann and Steer, this volume), membrane trafficking and secretion (Hwang and Yang, this volume; Malhó et al. 2005), self-incompatibility (De Graaf et al., this volume) and tube guidance (Johnson and Lord, this volume, and Higashiyama and Inatsugi, this volume).

However, the mechanisms which enforce and regulate the $[Ca^{2+}]_c$ gradient at the tube apex are still controversial (Hepler et al., this volume–Hwang and Yang, this volume). Apical influx of extracellular Ca^{2+} is required but there is an apparent discrepancy between internal Ca^{2+} measurements and external Ca^{2+} fluxes (Hepler et al., this volume) that suggest the existence of primary mechanisms to regulate the ion dynamics. The cell wall and/or internal stores (Malhó and Camacho 2004; Hepler et al., this volume and Sze et al., this volume) were suggested to play an important role. At the molecular level, GTPases have been proposed to act in this process as major signalling switches (Camacho and Malhó 2003; Zheng and Yang 2000) and a detailed discussion of this issue can be found in Chapter V. Phosphoinositides and signalling phospholipids are also emerging as powerful modulators of Ca^{2+}-mediated signals and crucial for the establishment and maintenance of tip growth. The role of these molecules can be found in Chapter VI.

2.2 Crosstalk of Signalling Pathways

CaM is a Ca^{2+} sensor known to modulate the activity of many proteins. In living pollen tubes CaM seems to distribute evenly (Moutinho et al. 1998) but a higher concentration of CaM-target molecules, possibly cytoskeletal elements, was suggested to exist in the sub-apical region. The actin distribution observed in the sub-apical region of pollen tubes (Hepler et al., this volume and Yokota and Shimmen, this volume) resembles the V-shaped col-

lar reported for CaM binding (Moutinho et al. 1998) and thus an interaction between CaM and actin has been hypothesized. This interaction could be dependent on the levels of phosphatidylinositol (4,5)-bisphosphate (Desrivières et al. 2002), thus linking CaM to the phosphoinositide signaling pathway (Zárský, this volume).

Although CaM distributes evenly, Rato et al. (2004) found that CaM activity is higher in the apex of growing tubes and the area of higher activity superimposes to a considerable degree with the tip-focused $[Ca^{2+}]_c$ gradient. Furthermore, it was found that CaM activity oscillates with a period similar to $[Ca^{2+}]_c$ (40–80 sec). We have also shown, as with the manipulation of $[Ca^{2+}]_c$ in the apex (Malhó and Trewavas 1996), that a decrease in CaM levels in one side of the apical dome led to growth axis reorientation to the opposite side. This clearly involves CaM in the molecular events that control pollen tube guidance (Hepler et al., this volume; Johnson and Lord, this volume). CaM might also participate in a feed-back regulation of Ca^{2+} stores (Sze et al., this volume). CaM can achieve regulation of Ca^{2+} stores and Ins(1,4,5)P_3 receptors (reviewed in Malhó and Camacho 2004) suggesting that CaM may allow both feedback control of membrane receptors and integration of inputs from other signaling pathways.

In addition to a role for Ca^{2+} in the control of CaM activation, Rato et al. (2004) provided evidence that a cAMP signalling pathway is involved. A cAMP-dependent signalling pathway in pollen was recently shown (Moutinho et al. 2001) and cAMP levels were found to be approximately uniform in the pollen tube cytosol but showing transient increases in the apical region upon reorientation and apical perturbations. CaM thus emerges as a strong candidate to integrate signals between Ca^{2+} and cAMP signalling pathways. Rato et al. (2004) found also that pharmacological modulation of cAMP levels caused equivalent changes in CaM activity suggesting that the activation of downstream targets of cAMP is involved in the regulation of CaM activity, possibly through $[Ca^{2+}]_c$.

The actin cytoskeleton and the secretory apparatus are putative candidates for a cross-regulation between signalling pathways. A growing body of evidence implicates CaM as an important receptor linking changes in Ca^{2+} with cytoskeletal function (Yokota and Shimmen, this volume) and Rato et al. (2004) found that a decrease in CaM levels on one side of the apical dome results in a decrease of secretory activity and reorientation. Diminishing cAMP levels mimicked this effect while an increase of cAMP (which augments CaM activity) promoted secretion. These data further support the claim for a close relationship between Ca^{2+} – CaM and intracellular cAMP in the control of pollen tube growth. Phosphoinositides and phospholipids have also been reported to modulate the actin cytoskeleton and apical secretion. Monteiro et al. (2005a, 2005b) reported that PIP_2, Ins(1,4,5)P_3 and phosphatidic acid (PA) regulate tip growth through a multiple pathway system involving coordinated regulation of $[Ca^{2+}]_c$, endo/exocytosis

and vesicular trafficking. In its turn, the levels of intracellular PIP_2 seem to be regulated by ROP GTPases (Kost et al. 1999; Hwang and Yang, this volume) thus linking ROP signalling to microfilament dynamics and apical secretion.

2.3 The Role of Cyclic Nucleotides – an Open Question

The role of cyclic nucleotides (CNs) in plant cell signaling has been clouded by many uncertainties and much controversy. In pollen, cloning of a putative adenylyl cyclase (AC) revealed common motifs with its fungal counterpart (Moutinho et al. 2001) but also with proteins involved in disease responses. cAMP is believed to be involved in such responses (Cooke et al. 1994) and parallels between pollen tube growth in the style and fungal hyphal infection are frequently drawn. The transformation with antisense oligos directed against the pollen AC cDNA or treatment with antagonists of AC caused disruption of pollen tube growth suggesting a requirement for continued cAMP synthesis. This was supported by the imaging of cAMP in growing pollen tubes where it was observed that forskolin, an AC activator, transiently increased cAMP whilst dideoxyadenosine, an inhibitor of ACs, caused a temporary decline (Moutinho et al. 2001).

The targets of the cAMP signaling pathway are still largely unknown. cAMP may be involved in the regulation of Ca^{2+} levels (Volotovski et al. 1998) and tubes submitted to different treatments that putatively affect the levels of cAMP experienced $[Ca^{2+}]_c$ transients (Malhó et al. 2000). After caged release of an estimated $\sim 1–2\ \mu M$ cAMP, growth rates temporally declined and reorientation of the tube growth axis occurred. This was accompanied by a transient $[Ca^{2+}]_c$ elevation in the apex but not in the sub-apical region. In contrast, the addition of external cAMP led to a complete growth arrest and a $[Ca^{2+}]_c$ increase in the apical and sub-apical regions. When compared to the release of a small concentration of cAMP after flash photolysis, this suggests a toxic effect and a drastic perturbation of ion fluxes. Thus, a cAMP pathway might be involved in pollen tube reorientation that most likely acts together with Ca^{2+} to control tube reorientation. Interestingly, experiments with a membrane-permeable version of cGMP diffusing from a micro-needle placed near the tip of growing pollen tubes, failed to cause a visible response (Moutinho et al. 2001). Prado et al. (2004) suggested that cGMP could participate in the signaling cascade that affects growth regulation. However, the authors acknowledged that the drugs used in this work clearly disrupted growth regulation, so the pleiotropic responses made it difficult to isolate or test its specificity. Further experimentation is required to evaluate the role of other CNs in pollen tube growth and reorientation.

2.4
Signalling Cascades – the Example of Protein Kinases

Phosphorylation cascades regulated by protein kinases and phosphatases represent primary, downstream transduction routes. In plants, many of the kinases identified belong to the Ca^{2+}-dependent protein kinase or CaM-like domain protein kinase (CDPK) superfamily (Harmon et al. 2000). Among other targets, CDPKs have been suggested to be involved in the regulation of tension. Myosin and/or actin cross-linking proteins are likely to be the principal regulator(s) of tension within the actin network (Yokota and Shimmen, this volume), and thus the principal targets of CDPKs. These proteins have also been found to influence the activity of a Ca^{2+} – CaM dependent pump in the endoplasmic reticulum (Hwang et al. 2000). In pollen tubes, a CDPK antibody was found to co-localize with F-actin (Putnam-Evans et al. 1989) while Moutinho et al. (1998a) reported the presence of a CDPK with a higher activity in the apical region of growing cells. When apical Ca^{2+} values were modulated (using caged-probes), the activity of this CDPK changed accordingly and induced reorientation of the growth axis. This suggests that the protein kinase activity measured by Moutinho and co-workers and the one reported by Putnam-Evans et al. (1989) correspond to different members of this family. However, Putnam-Evans et al. (1989) did not show data of how the antibody distributes in the apical region and, therefore, a direct comparison between the different results cannot be established. More definitive conclusions require further results.

Protein kinases crosstalk with other signalling pathways, namely through 14 : 3 : 3 proteins. 14-3-3 proteins bind to several CDPKs and also to sites in proteins that have been phosphorylated by CDPK (Cheung et al. 1999). One family of 14 : 3 : 3 proteins, the so-called fusicoccin receptor, controls plasma membrane ATPase activity through a specific Ca^{2+}-dependent and fatty acid-dependent protein kinase (Van der Hoeven et al. 1996). In pollen, 14-3-3 proteins have been recently identified and found to affect ATPase activity (Pertl et al. 2001; Sze et al., this volume). Further studies in apical growing cells to explore additional 14-3-3 targets are thus required.

Mitogen Activated Protein Kinases (MAPKs) have also been reported in pollen (Wilson and Heberle-Bors 2000). Activated MAPKs may translocate to the nucleus and phosphorylate transcription factors, target cytosolic proteins such as other kinases or cytoskeletal-associated proteins and regulate osmosensing, all crucial functions in tip growth. In *Papaver rhoeas* pollen tubes, MAPKs were found to be associated with the self-incompatibility response and the triggering of an apoptotic mechanism (De Graf et al., this volume) suggesting that these proteins might participate in a wide range of signalling responses.

3
Targets for Signalling – the Example of the Secretory Pathway

Pollen tube elongation is achieved through the continuous apical fusion of vesicles containing wall precursors. This process dictates growth speed, changes in growth axis, delivery and recycling of membrane components and is, most likely, an important gateway for extracellular signals. It is therefore a convergent point for signalling pathways. We recently discuss this issue in the first book of this "Plant Cell Monograph" series (Malhó et al. 2005) and the reader is referred to it for detailed information.

With the significant advances in molecular analysis and fluorescent probes, the mechanisms that drive vesicle trafficking start to be unveiled. Measurements of endo/exocytosis in growing pollen tubes became recently possible with the introduction of FM dyes such as 1-43 [*N*-(3-triethylammoniumpropyl)-4-4-dibutylamino-styryl-pyridinium dibromide]. Using FM 1-43 and confocal microscopy, we observed that growing pollen tubes exhibit a fluorescence hotspot at the apex (Camacho and Malhó 2003). This hotspot has an inverted cone shape that confirms the labelling of the apical secretory vesicles and partly superimposes with the $[Ca^{2+}]_c$ gradient indicating that $[Ca^{2+}]_c$ is a regulator of the coupling between growth and endo/exocytosis.

Modulation of intracellular GTP levels also resulted in a modification of apical secretion dynamics (Camacho and Malhó 2003) suggesting that cell growth is not strictly dependent of a Ca^{2+}-mediated stimulation of exocytosis. This is in agreement with the results of Roy et al. (1999) using the Yariv reagent who concluded that a Ca^{2+}-dependent exocytosis served mainly to secrete cell wall components. It is likely that GTPases play an active role in endo/exocytosis by coupling the actin cytoskeleton to the sequential steps underlying membrane trafficking at the site of exocytosis (Hwang and Yang, this volume). This hypothesis is supported by the recent discovery of Rho and Rab effectors of the exocyst complex and their importance for pollen tube growth (Cole et al. 2005; Zárský et al., this volume). As discussed above (§ 2.2), changes in the intracellular levels of phosphoinositides, CaM and cAMP also modify growth rate and orientation though modulation of apical secretion (Monteiro et al. 2005a; Rato et al. 2004). Although the targets of these molecules are presently undetermined, it is likely that they achieve their function by modulation of ion channels (Sze et al., this volume) and actin-binding proteins (Yokota and Shimmen, this volume).

3.1
Apical Secretion: is "Kiss-and-Run" a Likely Explanation?

Quantitative data have revealed that the quantity of membrane delivered by exocytosis at the tube apex is clearly in excess (up to 9×) for the cell

growth rate (Steer and Steer 1989) indicating that, coupled to secretion, an underlying recycling process must take place. In frozen-fixed pollen tubes, Derksen et al. (1995) observed the presence of a collar of coated vesicles in the sub-apical region, thus suggesting their involvement in the retrieval of excess secreted membrane material. Clathrin coated vesicles are, however, not visible in the apex of such tubes. Furthermore, observations using contrast enhanced video microscopy revealed that full endocytic or exocytic events do not seem to occur (Derksen et al. 1995; Ovecka et al. 2005).

These data led us to propose recently that a rapid endocytosis mechanism might occur in the apex of rapidly growing pollen tubes (Camacho and Malhó 2003; Monteiro et al. 2005a,b; Malhó et al. 2005). The rapid endocytosis mechanism is a Ca^{2+}-dependent process, coupled to exocytosis that requires GTP hydrolysis and dynamin but not clathrin (Artalejo et al. 1995). The importance of the dynamin-like protein ADL1C for plasma membrane maintenance during pollen maturation has been demonstrated (Kang et al. 2003). Rapid endocytosis is characterized by the formation of a small and short-lived pore, which limits the size of particles that can be released/incorporated. Indeed, we found that without artificial permeabilization, growing pollen tubes of *Agapanthus umbellatus* were unable to incorporate dextrans larger then 4 kDa (Camacho and Malhó, unpublished data). A rapid endocytosis mechanism is also compatible with extreme fast delivery of wall material required during in vivo where growth rates far exceed those observed in vitro. Such mechanism could operate in parallel to the "conventional" coated-vesicle system where endo- and exocytosis are uncoupled. The uncoupling would be favoured when cell growth is arrested or it is slowdown (apical $[Ca^{2+}]_c$ is lower). In such a system, the formation of a full endocytotic vesicle allows the incorporation of larger molecules and protoplasts obtained from pollen tubes (where $[Ca^{2+}]_c$ is low) showed indeed a capacity to incorporate molecules up to 20 kDa. In barley protoplasts, Homann and Tester (1997) have also reported the existence of two exocytotic modes, a Ca^{2+}-dependent and a GTP-binding-dependent mode. The uncoupled mechanism could operate in sub-apical regions at $[Ca^{2+}]_c$ resting levels and at the apex in situations where the cell needs to interpret extracellular cues and reorient the growth axis. These invariably involve reduction of growth rates, localized changes in Ca^{2+}, redirectioning of the actin cytoskeleton and target sites for fusion. A putative rapid endocytosis mechanism would be confined to the extreme apex operating during the stages of fast-directed growth. Proteins involved in vesicle traffic events such as SNAREs, SNAPs, syntaxins and VAMP-like proteins have been identified in the *Arabidopsis* genome (Twell et al., this volume) revealing that plant cells are equipped with a molecular machinery equivalent to animals.

3.2
Functional Zonation in the Pollen Tube – Challenging an Old Perspective

Underlying the structural zonation described for pollen tubes is the idea of a continuous flux of components (vesicles, proteins, wall precursors) towards the apex. The resulting paradigm is then, if a molecule is important for tip growth, its concentration should be higher in the apex. However, technological advances and re-evaluation of older data revealed that this is a too simplistic view. E.g., it is now generally accepted that early mapping of actin, myosin and calmodulin distribution were flawed by experimental artefacts.

Observations of vesicle dynamics using FM dyes suggest that most of the secretory vesicle processing (movement, fusion, recycling) is confined to the sub-apical and apical region (Malhó et al. 2005; Castanho-Coelho and Malhó, unpublished data). Fusion will take place mainly at the apex and recycling at the sub-apex forming a sort of spatially restricted loop. Interestingly, such loop resembles the electric dipole described for these areas (Feijó et al. 1994), two events that can enforce a positive feed-back and thus maintain a highly polarized domain. $[Ca^{2+}]_c$ oscillations are also confined to this area (Hepler et al., this volume) and recent findings on GTPase localization (Hwang and Yang, this volume), the presence of an exocyst complex (Zárský et al., this volume) and actin dynamics (Twell et al., this volume) further suggest that the apical and sub-apical regions contain all the necessary enzymatic machinery to maintain growth (protein synthesis and turn-over, lipid metabolism, assembly of complexes, etc.). This challenges the idea of the required continuous flux of components and raises new questions about the physiology and biochemistry of the pollen tube.

4
Perspectives

The different chapters of this book outline the main questions of the pollen tube research field and point towards new challenges. Perhaps the most interesting is to explore the potential of the different "-omics" (genomics, proteomics, transcriptomics, metabolomics) for cell biology studies. We now have the possibility to pinpoint genes and proteins of interest, study their expression, localization and functional role. In an immediate future this implies perfectioning techniques for micromanipulation and imaging of *Arabidopsis* pollen tubes. Conversely, molecular biology tools must be extended to other species, namely those of commercial interest. For example, conducting membrane trafficking studies in such small but dynamic cells is clearly difficult and little data can be found in the literature (Cheung et al. 2002). It is also foreseen that future work will resort to in vivo systems in order

to fully understand the fertilization process. Although at a morphological level, pollen tubes grown in vitro mimic those grown in vivo, there are many evidences that significant differences must exist at a physiological and biochemical level.

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Pollen Development, a Genetic and Transcriptomic View

David Twell[1] (✉) · Sung-Aeong Oh[1] · David Honys[2,3]

[1]Department of Biology, University of Leicester, Leicester LE1 7RH, UK
twe@le.ac.uk

[2]Institute of Experimental Botany AS CR, Rozvojová 135, CZ-165 02 Praha 6, Czech Republic

[3]Department of Plant Physiology, Faculty of Sciences, Charles University, Viničná 5, CZ-128 30 Praha 2, Czech Republic

Abstract The haploid gametophyte generation occupies a short but vital phase in the life cycle of flowering plants. The male gametophyte consists of just two or three cells when shed from the anthers as pollen grains. It is this functional specialization that is thought to be a key factor in the evolutionary success of flowering plants. Moreover, pollen development offers an excellent model system to study many fundamentally important biological processes such as polarity, cell fate determination, cell cycle regulation, cell signaling and mechanisms of gene regulation.

In the first part of this chapter we review the progress achieved through genetic analysis in identifying gametophytic mutants and genes required for key aspects of male gametogenesis. In the second part we discuss recent advances in genome-wide transcriptomic studies of haploid gene expression and a critical evaluation of data treatment methods. Finally we provide a perspective of the impact of these data on future strategies for understanding the gametophytic control of pollen development.

1 Introduction

During the past decade there have been significant advances in the genetic and genomic technologies exploiting *Arabidopsis thaliana* as a model. These include completion of the *Arabidopsis* genome sequence (Arabidopsis Genome Initiative 2000), establishment of public databases of large numbers of cDNAs (Seki et al. 2002) and sequenced insertion site mutants (Alonso et al. 2003), extensive transcriptomic data sets (Zimmermann et al. 2004) including those for the "pollen transcriptome" (Honys and Twell 2004; Pina et al. 2005), as well as new "pollen-specific" research tools (Johnson-Brousseau and McCormick 2004). Fuelled by these rapidly expanding shared resources, research in pollen development has made substantial progress. Here we focus on advances in our understanding of the gametophytic control of postmeiotic pollen development from free microspores up to the point of mature pollen in the model *Arabidopsis*. Several other recent reviews provide a wider discussion of other important features of male fertility and pollen development that involve diploid cells of the sporophyte, such as anther differentiation, meiotic

division, and the influence of the tapetum on pollen development and pollen wall patterning (Ma 2005; McCormick 2004; Scott et al. 2004).

2 Pollen Development

Pollen development begins in the young anther locules and consists of two major phases – microsporogenesis and microgametogenesis. The primary sporogenous layer gives rise to the microsporocytes or meiocytes. Meiotic division of the diploid microsporocytes produces tetrads of four haploid microspores enclosed within thick callosic walls, which are then separated into individual microspores by an enzyme complex (callase) secreted by the tapetum (Fig. 1). Microspore development is accompanied by progressive vacuole biogenesis, fusion and fission events (Owen and Makaroff 1995; Yamamoto et al. 2003). In association with vacuole morphogenesis and microspore expansion, the microspore nucleus becomes polarised to an eccentric position against the microspore wall. The polarised microspores then undergo asymmetric division at pollen mitosis I (PMI) giving rise to bicellular pollen grains.

Pollen mitosis I is an intrinsically asymmetric division that gives rise to two daughter cells with completely different structures and cell fates (Twell et al. 1998). The large vegetative cell has dispersed nuclear chromatin and constitutes the bulk of the pollen cytoplasm. In contrast, the smaller generative cell has condensed nuclear chromatin and contains relatively few organelles and stored metabolites. Whereas the vegetative cell exits the cell cycle at G_1 phase, the generative cell remains division-competent and completes pollen mitosis II (PMII) to form the two sperm cells. Asymmetric cytokinesis following PMI effectively seals the fate of the smaller generative cell and possesses two special features in that: (1) no preprophase band of microtubules marks the future division plane, and (2) a unique curved cell plate is formed to enclose the generative nucleus. Two general models have been proposed to account for differential cell fate arising from asymmetric division at PMI (Eady et al. 1995). Both assume that vegetative cell gene expression is the default pathway resulting from the accumulation of gametophytic factors, and provide alternative mechanisms to explain how vegetative cell-specific genes are repressed in the generative cell. In essence gametophytic factors may be simply excluded from the generative cell pole, or hypothetical generative cell repressors at the generative cell-pole may block vegetative cell-specific gene expression. However, it is possible that a combination of both mechanisms operate to specify and reinforce generative cell fate.

After pollen mitosis I, the generative cell migrates inward resulting in a “cell within a cell” structure, enabling gamete transport within the pollen tube. Generative cell migration follows degradation of the hemispherical cal-

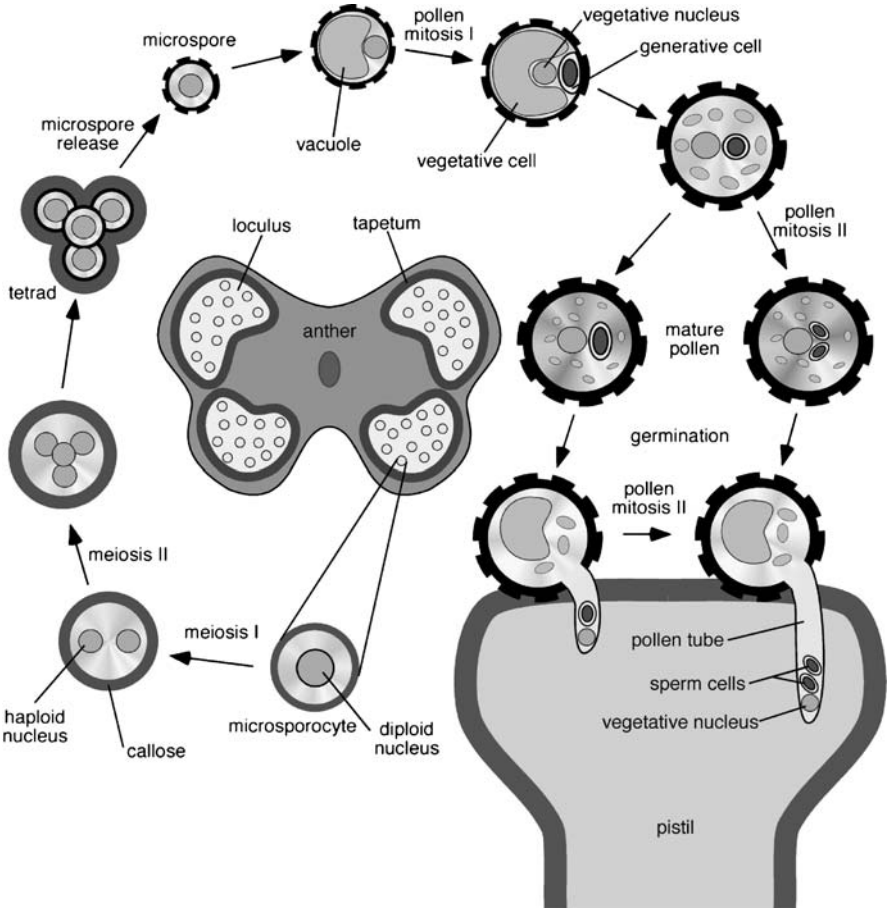

Fig. 1 Morphological stages of microsporogenesis and microgametogenesis. During microsporogenesis, microsporocytes undergo two nuclear divisions at meiosis followed by cytokinesis to produce a tetrad of four haploid microspores. During microgametogenesis, microspores undergo two stereotypical mitotic divisions, pollen mitosis I and pollen mitosis II to produce bicellular (70% of species) or tricellular pollen grains (e.g., *Arabidopsis*). In species with bicellular pollen grains, pollen mitosis II occurs in the growing pollen tube within the pistil

lose wall that separates the vegetative and generative cells. This presumably involves targeted secretion of β(1-3)-glucanases. Subsequently, the generative cell forms an elongated, or spindle-like shape that is maintained by a cortical cage of bundled microtubules (Cai and Cresti, this volume). Pollen mitosis II takes place within a membrane bound compartment of the vegetative cell cytoplasm and a physical association is established between the gametic cells and the vegetative nucleus known as the male germ unit (MGU). The MGU exists in both bicellular and tricellular pollen system and is thought

to be important for the coordinated delivery of the gametes and sperm cell fusion events (Dumas et al. 1998). In *Arabidopsis* the MGU is first assembled in tricellular pollen (Lalanne and Twell 2002). During pollen maturation the vegetative cell accumulates carbohydrate and/or lipid reserves required for the demands of plasma membrane and pollen tube wall synthesis (Pacini 1996). Pollen grains are usually strongly dehydrated when finally released from the anthers. The accumulation of sugars and amino acids as osmoprotectants, including disaccharides and proline or glycine-betaine, is thought to protect vital membranes and proteins from damage during dehydration (Schwacke et al. 1999).

2.1 Gametophytic Mutants Affecting Pollen Development

Mutants affecting pollen development provide excellent material to analyze processes of cell fate specification and cellular function (Sects. 2.1.1–2.1.4). There have been three major screening strategies to isolate gametophytic mutants and genes affecting pollen development in *Arabidopsis*; gametophyte-targeted forward genetic screens, gametophyte-directed reverse genetic screens and non-gametophyte-directed reverse genetic screens.

Male gametophyte-targeted forward genetic screens have identified altered pollen phenotypes caused by chemical or physical mutagens (Chen and McCormick 1996; Park et al. 1998; Grini et al. 1999), or by insertional mutagens of T-DNA (Bonhomme et al. 1998; Howden et al. 1998; Johnson et al. 2004) or transposon origin (Lalanne et al. 2004b). Disrupted genes have been identified by map-based cloning (Twell et al. 2002; Rotman et al. 2005; Oh et al. 2005) or by generating flanking sequence tags (Lalanne et al. 2004a,b; Johnson et al. 2004). Non-gametophyte-directed reverse genetic approaches have in several cases uncovered unexpected gametophyte-defective phenotypes caused by mutations in genes of interest (Robertson et al. 2004; Niewiadomski et al. 2005; Dettmer et al. 2005). While this approach is generally not gametophyte-targeted, prior analysis of the expression patterns of genes of interest can direct research toward testing gametophytic function. For example gametophyte functions for the tyrosine phosphatase *AtPTEN1* (Gupta et al. 2002; Yokota and Shimmen, this volume) were discovered by RNAi-directed down-regulation in pollen using the vegetative-cell-specific *LAT52* promoter (Twell 1992).

At the time of writing, we were able to compile data on 45 gametophytic mutants that show defects during post-meiotic male gametophyte development before pollen release (Table 1). The most dominant class of 28 mutants displays pollen cell death phenotypes at various developmental stages. A second major class of nine mutants show specific division-related phenotypes either affecting microspore division (*scp*, *gem1,2*, *tio*) or generative cell division (*duo1,2,3,4,5*). Different approaches have led to the identification of 14 genes

Table 1 Arabidopsis gametophytic mutants and genes that affect pollen development

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
abnormal gametophytes	*AGM*	Pollen degenerates at late microspore stage	At5g44860	V	Putative transmembrane protein	Microspore development and/or division	Sorensen et al. 2004
arabidopsis dynamin-like 1C	*ADL1C*	Pollen abortion during maturation	At1g14830	V	Dynamin-like protein	Pollen plasmamembrane maintenance, cell wall synthesis	Kang et al. 2003
arabidopsis phosphatase and tensin homologue	*AtPTEN*	Pollen death after pollen mitosis II	At5g39400	V	Phosphatase and tensin homologue [Tyrosine/ PIP3 phosphatase]	Pollen maturation	Gupta et al. 2002
arabidopsis H+-ATPase 3	*AHA3*	Aborted mature pollen	At5g57350	V	Plasma membrane H+-ATPase	Microspore/pollen nutrient transport	Robertson et al. 2004
arabinogalactan protein1[AtBCP1]	*AGP1*	Pollen aborts at bicellular stage	At1g24520	V	Arabinogalactan protein	Tapetum, microspore and bicellular pollen viability	Xu et al. 1995
both male and female defective (1,2,3)	*BOD*	Pollen arrested at bicellular stage with pleiotropic effects	Chr 1 ~ 108/18/88 cM	—	—	Pollen maturation after pollen mitosis I	Grini et al. 1999
duo pollen1	*DUO1*	Bicellular pollen: generative cell fails to enter pollen mitosis II	At3g60460	V	R2R3 MYB transcription factor (MYB125)	Regulator of gene expression required for mitotic entry	Durbarry et al. 2005 Rotman et al. 2005
duo pollen2	*DUO2*	Bicellular pollen: generative cell arrested at prometaphase	Chr 5 ~ 54 cM	—	—	Required for mitotic progression	Durbarry et al. 2005

Table 1 (continued)

Mutant [a,b]	Gene [b]	Mutant Phenotype	Map position	Status [c]	Protein identity	Function	Refs.
duo pollen3	*DUO3*	Bicellular pollen: generative cell fails to enter pollen mitosis II	Chr 1 ~95 cM	—	—	Required for mitotic entry	Durbarry & Twell, unpublished
duo pollen4	*DUO4*	Pollen fails to enter or complete generative cell division	Chr 4 ~75 cM	—	—	Generative cell morphogenesis and cell cycle progression	Twell 2002; Durbarry & Twell, unpublished
duo pollen5	*DUO5*	Pollen fails to enter or complete generative cell division	Chr 4 ~93 cM	—	—	Generative cell morphogenesis and cell cycle progression	Twell 2002; Durbarry & Twell, unpublished
emotionally fragile pollen1	*EFP1*	Pollen shows diffuse callosic staining	—	—	—	Repression of callose synthesis during pollen maturation	Johnson et al. 2001
female gametophyte3	*FEM3*	Aborted mature pollen	Chr 1 ~84 cM	—	—	Pollen viability	Christensen et al. 1998
gemini pollen1	*GEM1*	Twin-celled and binucleate pollen: abnormal divisions at pollen mitosis I	At2g35630	V	MAP215 family of microtubule associated proteins	Microspore polarity and cytokinesis through microtubule organisation	Park et al. 1998; Park and Twell 2001; Twell et al. 2002

Table 1 (continued)

Mutant [a,b]	Gene [b]	Mutant Phenotype	Map position	Status [c]	Protein identity	Function	Refs.
gemini pollen2	*GEM2*	Similar to *gem1*, but less severe	Chr 5 ~ 95 cM	—	—	Control of microspore polarity and cytokinesis	Park et al. 2004
gametophytic factor3	*GFA3*	Vacuolated mature pollen	Chr 2 ~ 73 cM	—	—	Vacuole morphogenesis	Christensen et al. 1998; Feldmann et al. 1997
gametophytic factor4	*GFA4*	Aborted mature pollen	Chr 3 ~ 98 cM	—	—	Pollen viability	Christensen et al. 1998; Feldmann et al. 1997
gametophytic factor5	*GFA5*	Aborted mature pollen	Chr 4 ~ 25 cM	—	—	Pollen viability	Christensen et al. 1998; Feldmann et al. 1997
glucose-6-phosphate translocator1	*GPT1*	Aborted pollen. Reduced lipid bodies, vesicles and vacuoles. Defects at tri-cellular stage	At5g54800	V	glucose-6-phosphate translocator	Glc6P import for starch, fatty acid biosynthesis and carbon for OPPP	Niewiadomski et al. 2005
gift wrapped pollen 1	*GWP1*	Pollen contains internal callose tube-like structures	Chr 2 ~ 50 cM	—	—	Regulation of callose synthesis	Johnson et al. 2001

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
gift wrapped pollen 2	*GWP2*	Pollen contains internal callose tube-like structures	—	—	—	Regulation of callose synthesis	Johnson et al. 2001
male germ unit malformed(1,2)	*GUM(1,2)*	Male germ unit does not form: vegetative nucleus separated from two sperm cells	Chr 4 ~ 24.2 cM	—	—	Male germ & stability unit assembly	Lalanne & Twell 2002
halfman	*HAM*	Pollen degenerates during bicellular stage	At4g28490 -28830	V	~ 150 kb deletion including 38 predicted genes	Pollen maturation after pollen mitosis I	Oh et al. 2003
hapless5	*HAP5*	Aborted mature pollen	At1g30450	T	Cation-chloride cotransporter	Ion homeostasis during development	Johnson et al. 2004
hapless12	*HAP12*	Aborted mature pollen	At4g36900	T	Contains AP2 domain (RAP2.10)	Transcriptional regulator of pollen gene expression	Johnson et al. 2004
hapless16	*HAP16*	Slighltly collapsed mature pollen	—	—	—	Pollen maturation	Johnson et al. 2004
limpet pollen	*LIP*	Generative cell remains attached to pollen wall	—	—	—	GC internalisation	Johnson et al. 2001
male defective1	*MAD1*	Pollen arrested during bicellular development, some internal dividing walls	Chr 1 ~ 118 cM	—	—	Vegetative cell maturation and GC division	Grini et al. 1999

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
male defective2	*MAD2*	Pollen arrested during bicellular development, diffuse GC nuclei	Chr 1 ~ 84 cM	—	—	Pollen maturation and GC differentiation	Grini et al. 1999
male defective3	*MAD3*	Smaller mature pollen, GC arrested late during bicellular development, intine defects	Chr 1 ~ 104	—	—	Involved in intine synthesis required for pollen maturation and GC division	Grini et al. 1999
male germ unit displaced1	*MUD1*	Male germ unit displaced to the cortical cytoplasm in mature pollen	Chr 3 ~ 41.6 cM	—	—	Regulation of nuclear-cytoplasmic organisation	Lalanne & Twell 2002
male germ unit displaced2	*MUD2*	Male germ unit displaced to the cortical cytoplasm in mature pollen	Chr 2 ~ 71.7 cM	—	—	Regulation of nuclear-cytoplasmic organisation	Lalanne & Twell 2002
polka dot pollen	*PDP*	Pollen shows abnormal localised callose staining	—	—	—	Regulation of callose synthesis	Johnson et al. 2001
ungud(1,2)	*UNG(1,2)*	Aberrent microspore divisions and bicellular pollen abortion	—	—	—	Microspore and pollen development, division at pollen mitosis I	Lalanne et al. 2004b
ungud3	*UNG3*	Progressive cell death from microspore through bicellular pollen stages	At2g34550	T	Gibberellin-2-oxidase	Control of gibberellin levels required for cell development	Lalanne et al. 2004b
ungud4	*UNG4*	Smaller pollen showing bicellular and tricellular arrest	—	—	—	Bicellular pollen development	Lalanne et al. 2004b

Table 1 (continued)

Mutant[a,b]	Gene[b]	Mutant Phenotype	Map position	Status[c]	Protein identity	Function	Refs.
raring-to-go	*RTG*	Precocious germination of pollen within the anther locule	Chr 3 ~ 30 cM	—	—	Regulation of pollen hydration status	Johnson et al. 2001
sidecar pollen	*SCP*	Pollen with an extra vegetative cell: early symmetric microspore division	Chr 3 ~ 80 cM	—	—	Regulation of microspore cell cycle or polarity establishment	Chen & McCormick 1996
T-DNA transmission defective (6,17,38,40)	*TDT*	Pollen aborted at pollen mitosis I or in mature anthers	—	—	—	Microspore or pollen viability	Bohomme et al. 1998; Procissi et al. 2001
two-in-one	*TIO*	Microspores initiate, but fail to complete cytokinesis at pollen mitosis I	At1g50230	V	TIO: Homologous to FUSED-kinase family	Signalling role in phragmoplast expansion	Oh et al. 2005
Vacuolar-ATPase V1 subunit A	*VHA-A*	Pollen aborted at bicellular stage and later: Swollen ER cisternae	At1g78900	V	Vacuolar-ATPase V1 subunit A	pH homeostasis, secondary active transport, Golgi organization	Dettmer et al. 2005

[a] Only gametophytic mutants that show abnormal phenotypes detectable before pollen shed are included.
[b] Numbers in parenthesis refer to individual mutants or genes with the same mutant symbol prefix.
[c] Gene function status: V = verified by independent methods [alleles, KO, antisense, RNAi or complementation]; T = tagged gene(s) showing 100% linkage, but not yet independently verified.
— = not determined

responsible for male-gametophyte developmental mutations (Table 1). Half of these were identified through forward genetics, by positional cloning (*GEM1, DUO1, TIO*), or by isolating flanking sequence tags (*UNG3, AGM, HAP5, HAP12*). The other half were functionally identified using reverse genetics approaches (*AGP1, AtPTEN, ADL1C, AGM, AHA3, GPT1, VHA-A*). Proteins with a variety of cellular roles have been defined including those with roles in cell division (GEM1, DUO1, TIO) nutrition and metabolism (AHA3, GPT1), pH regulation and ion transport (VHA-A, HAP5), and other groups with roles in transcriptional regulation (DUO1, HAP12) or post-translational (AtPTEN, TIO) intracellular signaling events (Table 1).

Two forward genetic strategies have been adopted to identify gametophytic mutants affecting pollen development. First, by morphological screening of pollen from mutagenized plants using histochemical staining for DNA (Chen and McCormick 1996; Park et al. 1998) or callose (Johnson and McCormick 2001). Second, by analysis of pollen phenotypes in plants showing marker segregation ratio distortion (Bonhomme et al. 1998; Grini et al. 1999; Howden et al. 1998; Johnson et al. 2004; Lalanne et al. 2004a,b; Procissi et al. 2001). Morphological screens are labor intensive, but have proven effective in the identification of several important mutants with novel phenotypes (Table 1). These include mutants that disturb asymmetric cell division at pollen mitosis I (Chen and McCormick 1996; Twell et al. 1998, 2002; Oh et al. 2005), generative cell division (Grini et al. 1999; Durbarry and Twell 2005; Rotman et al. 2005), generative cell migration (Howden et al. 1998), the positioning and structure of the male germ unit (Lalanne and Twell 2002), callose wall deposition and the repression of pollen germination within the anther (Johnson and McCormick 2001).

2.1.1 Mutants Affecting Gametophytic Cell Divisions

sidecar pollen (*scp*) is a male-specific mutant that affects microspore division and cell patterning (Chen and McCormick 1996). Mutant *scp* microspores first undergo a premature symmetrical division, followed by asymmetric division of only one of the daughter cells to produce mature pollen with a supernumerary vegetative cell. Although polarity is not visibly expressed when *scp* microspores divide, polarity is not defective, since it is re-established in one daughter cell. *scp* provides evidence for the importance of coordinating the mitotic cell cycle with the expression of polarity. Although *SCP* remains to be identified, the mutant phenotype supports the hypothesis of an asymmetric distribution of polarity determinants before division in *scp* (Chen and McCormick 1996).

gemini pollen1 (gem1) affects male and female transmission and displays a range of microspore division phenotypes including equal, unequal and partial divisions (Park et al. 1998). In contrast to *scp*, symmetrical divi-

sions in *gem1* do not occur precociously and neither daughter cell completes a further division. Cell fate analysis in *gem1* supports the role of cell size or nuclear/cytoplasmic ratio as a factor in determination of cell fate (Park et al. 1998; Park and Twell 2001). *GEM1* was the first male gametophytic gene to be positionally cloned, revealing its identity to the MOR1 (Whittington et al. 2001). MOR1/GEM1 is homologous to the MAP215 family of microtubule-associated proteins that stimulate plus-end microtubule growth. MOR1/GEM1 is associated with interphase, spindle and phragmoplast microtubule arrays and plays a vital role in microspore polarity as well as cytokinesis (Twell et al. 2002). To understand the role of MOR1/GEM1 in more detail we have recently expressed a GFP alpha-tubulin fusion in developing pollen that will allow the analysis of microtubule dynamics in wild type and mutant genetic backgrounds (S-A. Oh, J.A. Johnson, D. Twell unpublished).

In the *two-in-one* (*tio*) mutant, microspores complete nuclear division but fail to complete cytokinesis resulting in binucleate pollen grains. In contrast to *gem1, tio* shows normal nuclear polarity before pollen mitosis I, but displays cytokinesis-specific defects including the formation of incomplete callosic cell plates that fail to expand (Fig. 2). TIO was recently identified as

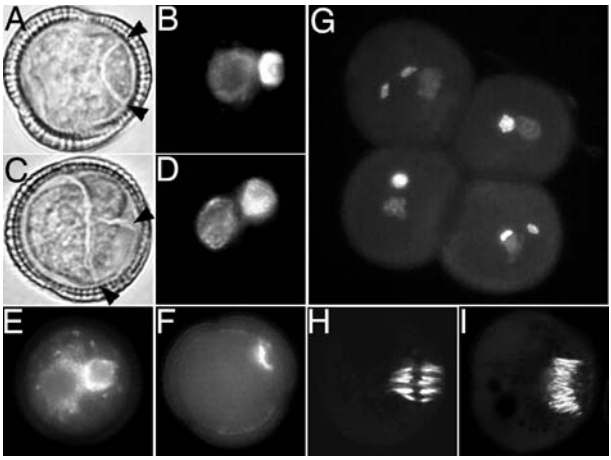

Fig. 2 Pollen phenotypes of *Arabidopsis* gametophytic mutants and GFP::tubulin expression (**A–D**) Bright field (**A**,**C**) and corresponding DAPI-stained (**B**,**D**) images of wild type (**A**,**B**) and *gem1* (**C**,**D**) pollen at early bicellular stage. Wild type pollen shows a typical hemispherical cell wall but *gem1* pollen grains often produce misplaced and branched internal cell walls. **E** DAPI-stained *tio* pollen with typical binucleate phenotype resulting from failed cytokinesis at pollen mitosis I. **F** *tio* pollen immediately after nuclear division at PMI stained with aniline blue showing an incomplete and transient callose cell plate. **G** Tetrad from heterozygous *duo1* mutant in the *quartet1* background showing 2 : 2 segregation for wild type and *duo1* mutant pollen. (**H**, **I**) Spindle (**H**) and phragmoplast microtubules (**I**) at pollen mitosis I visualised in transgenic tobacco microspores expressing GFP::TUA6

the plant homologue of the Ser/Thr protein kinase FUSED (Oh et al. 2005), which is a key component of the hedgehog signalling pathway in fruitflies and humans (Lum and Beachy 2004). TIO is localised to the phragmoplast midline where it has an essential role in centrifugal cell plate expansion. Although nuclear division is not affected in *gem1* and *tio* microspores, daughter nuclei do not divide further, strengthening the hypothesis that PMII depends on persistent cell fate determinants that may be sealed in the generative cell cytoplasm at PMI.

The *duo pollen* (*duo*) mutants complete normal asymmetric division at PMI, but fail to enter or complete generative cell division (Durbarry et al. 2005). Heterozygous *duo* mutants produce ~ 50% bicellular pollen containing a vegetative cell nucleus and a single "generative-like" cell with condensed nuclear chromatin (Fig. 2). DUO1 was recently identified as a novel R2R3 MYB protein specifically expressed in generative and sperm cells (Rotman et al. 2005). DUO1 represents the first germ line specific regulator to be identified and DUO1 homologues in maize, rice and tobacco all possess a supernumerary lysine signature in the R3 MYB domain that defines the DUO1 family (Rotman et al. 2005). Mutant generative cells in *duo1* pollen fail to enter PMII and enter a partial endocycle, suggesting that DUO1 may be a specific regulator of genes required for G2 to M-phase transition (Durbarry et al. 2005). In contrast, mutant generative cells in the *duo2* mutant do enter mitosis, but arrest at prometaphase suggesting the role of DUO2 in mitotic progression (Durbarry et al. 2005).

2.1.2 Mutants Affecting Pollen Cell Morphogenesis

Several interesting mutants have been isolated that affect pollen cell morphogenesis after PMI using different screens. In the *limpet pollen* (*lip*) mutant, identified in a T-DNA segregation distortion screen, the generative cell fails to migrate inward after PMI suggesting defects in mechanisms of generative cell engulfment. Moreover, the generative cell is able to divide to form two marginalized sperm cells outside the vegetative cell cytoplasm suggesting that positional information is not essential for generative cell division (Howden et al. 1998). The *mud* (*male germ unit displaced*) and *gum* (*germ unit malformed*) mutants were identified from morphogenesis screens as male-specific gametophytic mutants affecting the MGU (Lalanne and Twell 2002). *mud* pollen displays normal MGU assembly, but the MGU is displaced to an eccentric position in mature pollen. In *gum* pollen however, the MGU fails to assemble and the vegetative nucleus is separated from the two sperm cells. Double mutant analysis suggests that *GUM* acts upstream of *MUD* in a pathway required for MGU assembly and positioning. From independent morphological screens a range of mutants were isolated that show unusual callose staining (Johnson and McCormick 2001). These

include *raring-to-go* (*rtg*) that shows premature pollen hydration and germination within the anther locules. Other mutants producing excess callose in pollen grains, *emotionally fragile pollen* (*efp*), *gift-wrapped pollen* (*gwp*) and *polka dot pollen* (*pdp*), may be regulators of callose synthase and/or components pathways that normally repress callose production until pollen germination.

2.1.3
Segregation Ratio Distortion Screens for Male Gametophytic Mutants

Forward genetic screens using marker segregation ratio distortion have also been successful in identifying genes involved in microgametogenesis. These screens have mostly employed T-DNA or transposon insertion populations that harbor dominant antibiotic or herbicide resistance markers. For example, if an insertion inactivates an essential male gametophytic gene, then the ratio of resistant to sensitive progeny will deviate significantly below the expected 3 : 1 ratio toward 1 : 1. Such screens are inclusive in that mutants affecting both pollen developmental and progamic phases are recovered as well as those affecting female gametogenesis.

Insertional segregation ratio distortion screens have the advantage of straightforward identification of the mutated sequences. A number of these cause aborted pollen phenotypes that could arise from various mechanisms (Table 1). For example *abnormal gametophytes* (*agm*) encodes putative transmembrane protein; *vha-A*, a subunit of the vacuolar H^+ ATPase, tagged sequences in *hap5*, encode a putative AP2 domain transcription factor and in *hap12*, a cation-chloride transporter. The *ung3* mutant results in early bicellular pollen arrest (Lalanne et al. 2004b). Tagged sequences in *ung3* encode a GA-2-oxidase suggesting a role for GA in pollen development, as well as its recently discovered role in pollen tube growth (Singh et al. 2002).

A different segregation distortion screen for EMS-induced gametophytic mutants using multiple visible markers on chromosome 1 was devised by Grini et al. (1999). Three male-specific lines (*mad1*, *mad2*, *mad3*) showed defects during pollen development, and three other lines showed variable defects in both male and female gametophytes (*bod1*, *bod2*, *bod3*). Although most mutants showed pleiotropic effects, the most common phenotypes included pollen arrested at the bicellular stage. Interestingly, *mad1* also showed dividing walls at mid-bicellular stage reminiscent of phenotypes in *gem1* (Park et al. 1998).

Despite cloning successes complications can arise in insertion-based screens, and chromosomal rearrangements involving inversion, translocation and deletion have been reported (e.g., Tax and Vernon 2001). Many mutants with lethal phenotypes remain to be identified including several members of the, *both male and female defective*, *T-DNA transmission defective* and *ungud* mutant classes (Table 1). Failure to clone flanking sequences

could arise from such rearrangement of insertion sequences (Lalanne et al. 2004b; Johnson et al. 2004). For example in the *ham* mutant, insertion of a *Ds* element caused an approximately 150 kb deletion, hampering identification of the responsible gene(s) (Oh et al. 2003). Therefore it is important that the function of even apparently tagged mutants are independently verified by isolation of new alleles, complementation and/or by targeted down-regulation.

2.1.4
Non-gametophyte Targeted Identification of Male Gametophytic Genes

In an increasing number of cases, gametophytic roles have been discovered by characterising loss of function phenotypes caused by knockout or knock-down mutants in genes of interest. This approach has led to the identification of four genes, *AtPTEN, ADL1C*, *AHA3* and *GPT1*, with important roles during microgametogenesis (Table 1). In some cases progress was directed by knowledge of pollen-enhanced gene expression. For the dynamin-like *ADL1C*, gene expression profiles and promoter-GUS analysis hinted at a role for *ADL1C* in pollen. T-DNA insertions in *ADL1C* caused plasma membrane and cell wall defects that could suggest a role in for ADL1C in plasma membrane maintenance (Kang et al. 2003).

The requirement of two ATPases for pollen development was also demonstrated by isolating T-DNA insertion alleles. Knockout of a single member of the 11 plasma membrane proton H^+-ATPases, AHA3, leads to pollen abortion that suggests a role in secondary ion transport and microspore nutrition (Robertson et al. 2004). A T-DNA insertion in the A subunit of the vacuolar H^+ ATPase, VHA-A, leads to mutant pollen with curved and swollen Golgi cisternae suggesting a role in Golgi organization (Dettmer et al. 2005). The essential role of a plastidic glucose 6-phosphate/phosphate translocator (GPT1) in pollen was recently demonstrated by isolating knockout mutants in one of the two homologous GPT genes (Niewiadomski et al. 2005). *gpt1* mutant pollen shows reduced formation of lipid bodies and vacuoles essential for cell viability. The suggested role for GPT1 in pollen is to support Glc6P import into plastids as a source of carbon for starch and fatty acid biosynthesis, or as a starter for the oxidative pentose phosphate pathway.

2.2
Perspectives from Male Gametophytic Mutant Studies

Currently we know about 45 gametophytic mutants for which 14 corresponding genes have been identified (Table 1). Progress has been slowed because of the limitations of positional cloning and the effort required for large-scale segregation distortion screening. Saturation screening by segregation distortion remains a daunting task, since it is estimated that 180 000 T-DNA

insertions are required to achieve one insertion in every gene in *Arabidopsis* with 95% probability (Krysan et al. 1999). With the establishment of large FST (Flanking Sequence Tag) databases and corresponding germplasm stocks more than 80% of protein coding genes now have intron or coding regions FST matches likely to produce knockout mutations (Schoof et al. 2004). With such resources lines in hand, the total number of insertions that need to be screened to achieve saturation is more realistic and similar to the total number of annotated genes in *Arabidopsis*. However, it will remain difficult to identify essential genes like *TIO* with critical functions in both gametophytes (Oh et al. 2005) due to their extremely reduced transmission. Moreover, many genes with redundant roles will not be revealed until double and multiple mutant combinations can be assembled.

3 Pollen Transcriptomics

Characterisation of the complexity of male gametophyte gene expression has reflected the available methodologies. First, isozyme studies suggested significant overlap of gametophytic and sporophytic gene expression ranging from 60 to 72% (Tanksley et al. 1981; Pedersen et al. 1987). RNA hybridisation studies confirmed this overlap and suggested that *Tradescantia paludosa* and *Zea mays* pollen express ~ 20 000 to 24 000 individual mRNA sequences which was significantly lower than in roots (Willing et al. 1988). Subsequently, hybridisation studies of pollen cDNA libraries corrected the extent of gametophytic-sporophytic overlap suggesting that only 10% of pollen-expressed mRNAs may be pollen-specific (Stinson et al. 1987; Mascarenhas 1990).

Until recently, gene-by-gene characterization led to the identification of approximately 150 pollen-expressed genes from different species, with strong evidence for pollen-specific expression in about 30 (reviewed in Twell 2002). Moreover only 23 pollen-expressed genes were identified in *Arabidopsis*. The availability of new high-throughput technologies has enabled analysis of the haploid transcriptome on a global scale. Three initial studies, exploiting serial analysis of gene expression (SAGE) technology (Lee and Lee 2003) and 8K Affymetrix AG microarrays (Honys and Twell 2003; Becker et al. 2003), provided analyses for mature pollen based on approximately one-third of the *Arabidopsis* genome. Both approaches, however, gave similar overall views of pollen gene expression. The microarray studies led to the identification of 992 (Honys and Twell 2003) and 1587 (Becker et al. 2003) genes expressed in mature pollen of which 39%/10% were considered pollen-specific. Based on these limited genome-wide studies, estimates of pollen-expressed genes in *Arabidopsis* were between 3500 and 5500. The classification of pollen-expressed and pollen-specific genes into functional categories revealed sev-

eral over-represented functional groups (cell wall, metabolism, cytoskeleton and signaling) among the pollen-specific genes. Moreover, pollen-specific genes were in general much more highly expressed than corresponding non-specific pollen-expressed genes (Honys and Twell 2003).

Further refinement was enabled by the availability of Affymetrix 23 K *Arabidopsis* ATH1 arrays. Currently, there are three publicly available independent data resources. The first contains microarray data covering four stages of male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen and mature pollen) for ecotype Landsberg erecta (Honys and Twell 2004). The two remaining datasets were obtained from mature pollen grains from ecotype Columbia (Pina et al. 2005; Zimmermann et al. 2004). Apart from revealing the identity of the vast majority of male gametophyte-expressed genes, the major impact of these studies lies in the massively increased knowledge of the complexity and dynamics of haploid gene expression throughout single-cell development in plants (Honys and Twell 2004).

3.1 Quantification of Pollen-expressed Genes

Estimations of the number of genes active in the male gametophyte and their functional categorization are strictly dependent on data treatment, especially on the microarray normalisation algorithm[1] and the exclusive or inclusive treatment of detection calls[2] in replicates. We show how data treatment influences the outcome in Table 2. The use of the MAS4 algorithm in combination with the inclusive treatment of replicates can increase the number of expressed genes more than two-fold when compared to MAS5 using the exclusive approach. Therefore differentially normalised datasets cannot be reliably compared. Such variance however does not affect the correlation within differentially normalised data from one experiment; correlation coefficients were always above 0.99. This demonstrates that most of the variance is caused by low-abundance transcripts with uncertain expression. However, our analyses of four developmental stages showed that the MAS5 detection algorithm tended to eliminate a number of genes originally detected by MAS4 as expressed and which were experimentally confirmed as active. This was often the case even for abundant genes (B. Honysová and D. Honys unpublished results), highlighting the added value of the empirical MAS4 detection algorithm and comparative analysis.

[1] Two detection algorithms were compared: empirical MAS4 and statistical MAS5

[2] Exclusive approach: a gene must have the detection call PRESENT in all replicates to be scored as expressed, it eliminates non-reliable genes, the final number of identified genes in always smaller than in any replicate; inclusive approach: a gene must be called PRESENT in at least one replicate to be scored as expressed, it keeps non-reliable genes, the final number of genes is always higher than in any replicate.

Table 2 The effect of the normalisation algorithm used to identify the number of genes expressed in mature pollen in three independent experiments involving two different ecotypes (Section X)

Dataset[a]	Exclusive MAS4	MAS5	Inclusive MAS4	MAS5	MAX/MIN[b] fold change
Honys	**7,264**	4,774	11,071	6,930	2.32
Pina	6,965	5,039	11,238	**6,558**[c]	2.23
AtGE73-1,2	8,566	6,396	11,939	8,195	1.87
AtGE73-1,3	8,321	6,192	11,748	7,924	1.90
AtGE73-2,3	8,394	6,283	11,764	7,916	1.87
AtGE73-1,2,3	7,671	5,938	12,756	8,520	2.15
MAX/MIN[b] fold change	1.23	1.34	1.08	1.25	

[a] Dataset labelling: Honys, Landsberg erecta (duplicate; Honys and Twell 2004); Pina, Columbia (duplicate; Pina et al. 2005); AtGE73, Columbia (triplicate; Zimmermann et al. 2004); AtGE73-1,2, AtGE73-1,3, AtGE73-2,3, all possible combinations of replicate pairs; AtGE73-1,2,3, all three replicates.

[b] Ratio of the maximum/minimum values in appropriate row/column; bold values, published estimates and normalisation algorithms used (Honys and Twell 2004; Pina et al. 2005).

[c] Only nuclear genes were included; the published value (6,587) also includes mitochondrial and plastid-encoded genes.

On the contrary, when the same normalisation algorithm is applied to different datasets, the number of expressed genes is highly consistent, with a maximum difference of 25% (Table 2). This was also confirmed by comparison of correlation coefficients. All pairs of experiments except one, showed correlation coefficients above 0.94; the only exception was 0.91. This is significantly higher than in corresponding sporophytic experiments where values were within the range 0.78–0.99 (only experiments with more than one replicate were compared). Moreover, the differences observed between datasets did not correlate with ecotype for both gametophytic and sporophytic datasets. Therefore experimental conditions seemed to be more important than the ecotype itself and previously published speculations about significant pollen transcriptome differences between ecotypes (Pina et al. 2005) are not supported. Taken together, we can now estimate the total number of genes expressed in mature pollen to lie between 5000 and 7000 genes (see Table 2). We recommend analysis of expression data in pollen using both MAS4 and MAS5 algorithms. Normalized datasets based on both MAS4 and MAS5 can be accessed and downloaded at the Arabidopsis Gene Family Profiler (aGFP) site (http://aGFP.ueb.cas.cz).

3.2 Gametophytic-Sporophytic Overlap

Comparative analyses of the male gametophytic transcriptomes are being performed against an increasing number of sporophytic datasets. Unless otherwise stated, the following results were obtained using transcriptomic data from Landsberg erecta (four developmental stages, eight individual GeneChips; Honys and Twell 2004) as all corresponding samples originated from plants populations grown under identical conditions. Reference datasets were obtained from the NASCArrays database (Craigon et al. 2004), represent 62 experiments (154 individual chips) that provide transcriptomic data for seedlings, shoots, leaves, guard cells-enriched extracts, petioles, stems, hypocotyls, xylem, cork, root hair elongation zone, roots, inflorescences, young and old buds, siliques and cell suspension cultures[3]. All datasets were normalised together using the same algorithm[4].

Considering all developmental stages, 14 037 genes gave a positive expression signal in the male gametophyte. In individual stages the number of active genes gradually decreased from 11 615 (microspores, UNM) and 11 961 (bicellular pollen, BCP) through 8831 (tricellular pollen, TCP) to 7264 (mature pollen, MPG). Analysis and visualization of gametophytic-sporophytic overlap (Fig. 3A) demonstrates a relatively low number of strictly male gametophyte-specific genes (5.6% of all male gametophyte-expressed genes). This number is gradually decreasing as new sporophytic datasets emerge, especially those from more specialised tissues and individual cell types. The estimated number of strictly pollen-specific genes is therefore significantly lower than expected from previous studies (Tanksley et al. 1981; Pedersen et al. 1987; Stinson et al. 1987; Mascarenhas 1990; Honys and Twell 2003, 2004; Becker et al. 2003; Pina et al. 2005). However, those remaining male gametophyte-specific genes are characterised by very high expression signals, highlighting their importance and their potential as targets for functional analysis.

Male gametophyte gene expression can be divided into two major phases, early and late. 13 038 genes are expressed in microspores and bicellular pollen, while 9739 genes are expressed in tricellular and mature pollen. However there is significant overlap and the vast majority (8740 genes) are expressed in both phases (Fig. 3B). The division of genes into early and late groups enables more precise visualisation of possible overlaps of early and late male gametophytic genes with two subgroups of sporophytic genes, those expressed in above ground vegetative organs and those in roots. As an example of a deregulated tis-

[3] The complete list of dataset codes is available from authors.

[4] MAS4 detection algorithm, normalisation of all arrays to the median probe intensity level, calculation of model-based gene-expression values using Perfect Match-only model (dChip 1.3 software, http://www.dchip.org; Li and Wong 2001), exclusive treatment of replicates for identification of expressed genes

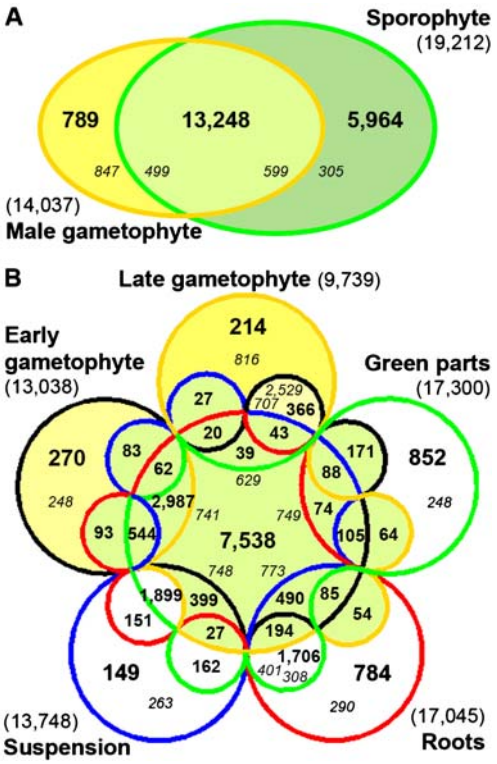

Fig. 3 Venn diagrams showing the quantitative overlap between male gametophytic and sporophytic transcriptome profiles. **A** Male gametophytic-sporophytic overlap. **B** Overlap of early pollen and late pollen transcriptomes with different sporophytic datasets represented by green parts (leaves, stems, petioles), roots and suspension cultures. The number of genes forming each segment is shown in bold. Numbers in italics show the average relative expression signal for genes in specific tissues within the particular segment

sue as well as another single cell type, suspension-cultured cells were included (Fig. 3B). From this analysis we can conclude that the late genes make a significantly greater contribution to the group of highly expressed pollen-specific genes. Late genes also represent the only dataset where the mean relative expression is higher for specific genes than for constitutive genes. The mean signal for gametophytic genes in the two late developmental stages (2529) is far higher than the mean signal within other genesets (mostly 300–500). These data also suggest that the early male gametophytic gene expression program is much more similar to that of the sporophyte.

Because of the gradually decreasing number of putative male gametophyte-specific genes, a new expression profile must be considered. This comprises non-specific pollen-expressed genes that show enhanced expression in the male gametophyte. Such pollen-enhanced genes were defined as genes with

maximum male gametophytic expression at least fivefold higher than the maximum expression in the sporophyte. We identified 1364 pollen-enhanced genes (9.7% of all male gametophytic genes), that were similarly distributed among late-pollen genes (1014 genes, 10.4% of late genes) and early-pollen genes (1084 genes, 8.3% of early genes). Within the late group these numbers resemble and thus confirm previously published estimates of preferential gene expression (Stinson et al. 1987; Mascarenhas 1990; Becker et al. 2003). Importantly, our analysis significantly extends this knowledge to include four stages of male gametophyte development.

3.3
Two Global Male Gametophytic Gene Expression Programs

Analysis of the overlap between gametophytic and sporophytic expression and the relative levels of expression in both generations clearly demonstrated marked differences. Moreover, there were striking differences between early and late male gametophytic transcriptomes that highlight the uniqueness of the late pollen transcriptome. The extent of such differences is most apparent when the expression profiles of all male gametophyte-expressed genes are visualised (Fig. 4A). Male gametophyte development is under control of at least two successive global gene expression programs, early and late, that should be precisely controlled at the transcriptional level. Out of approximately 1350 predicted *Arabidopsis* transcription factors (AGRIS web page: http://arabidopsis.med.ohio-state.edu/; Davuluri et al. 2003; Riechmann et al. 2000; Parenicova et al. 2003; Toledo-Ortiz et al. 2003), 612 were expressed in developing male gametophytes (542 early, 405 late). Of these, 49 were pollen-enhanced and only 27 were pollen-specific. These genes represent strong candidates for transcriptional regulators of male gametophyte development.

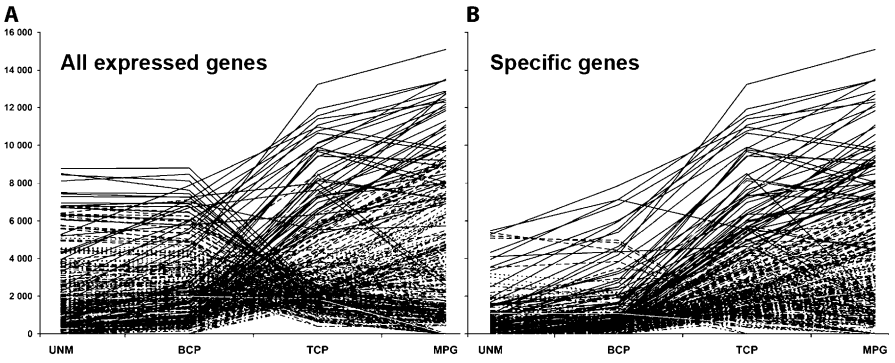

Fig. 4 Transcriptome profiles of all expressed genes (**A**) and male-gametophyte-specific genes (**B**) during male gametophyte development. There is a clear over-representation of highly-expressed late genes within the male-gametophyte-specific subset

Table 3 Correlation coefficients resulting from comparison of gametophytic and selected sporophytic transcriptomic datasets

Tissue	UNM [b]	BCP [b]	TCP [b]	MPG [b]	Chips [a]
UNM [b]	—	0.961	0.419	0.220	2
BCP [b]	0.961	—	0.565	0.333	2
TCP [b]	0.419	0.565	—	0.865	2
MPG [b]	0.220	0.333	0.865	—	2
Siliques	0.587	0.572	0.238	0.127	21
Embryo	0.593	0.558	0.166	0.073	18
Seedlings	0.468	0.470	0.214	0.117	10
Rosette leaves	0.449	0.446	0.185	0.093	28
Roots	0.549	0.550	0.166	0.116	10
Inflorescence	0.630	0.629	0.287	0.164	4
Young buds	0.576	0.576	0.256	0.143	3
Old buds	0.544	0.247	0.262	0.155	3
Suspension	0.545	0.541	0.278	0.164	5

[a] Number of individual GeneChips used for each analyses.
[b] uninucleate microspores, bicellular pollen, tricellular pollen, mature pollen.

The switch point between both developmental programs occurs prior to PMII (Fig. 4A). Taking into account the gradual decrease in the number of male-gametophyte-expressed genes, early genes, follow the general trend of reduction in complexity. The absence of most abundant early male gametophyte-specific genes in tricellular pollen datasets clearly demonstrates this switch. On the contrary, a number of very abundant late genes are activated after PMII and the majority of highly expressed late genes show pollen-specific expression patterns. This switch to late program genes supports the previously published uniqueness of the late male gametophytic transcriptome (Honys and Twell 2003; 2004). This uniqueness can be semi-quantified by comparison of correlation coefficients between gametophytic and sporophytic transcriptome datasets (Table 3). Moreover, gene expression early in gametophyte development is significantly more similar to that in the sporophyte than to late male gametophytic developmental stages. This could suggest a significant contribution of pre-meiotic gene expression to the early gametophyte expression profiles.

3.4 Functional Characterisation of Male Gametophytic Genes

Male gametophyte development is under the control of two very different developmental programs. To evaluate more thoroughly the divergence of these programs, the dynamics of the distribution of male gametophyte-

expressed mRNAs between gene function categories was analysed. Twelve gene function categories were defined as described previously (Honys and Twell 2003).

3.4.1 Transcription in Microspores

The distribution of microspore-expressed genes among functional categories was similar to that of rosette leaves and all genes on the ATH1 microarray (Fig. 5). Among rare exceptions were genes involved in signaling and stress-responses (~ 1% under-represented) and genes involved in protein synthesis and transport (~ 1% over-representation). More striking differences were found when the distribution of expressed genes was represented according to their relative expression levels. This led to a dramatic expansion in the contribution of genes involved in protein synthesis from 5% to 14%. This highlights the importance of the protein synthesis program initiated early during male gametophyte development.

With some exceptions (e.g., some transcription factors and receptor proteins), we can expect many gametophytic genes with important functions to be relatively highly expressed. The contribution of such genes is more easily visualized when only mRNAs forming the high-abundance class[5] are considered (2197 genes). Within the high abundance class, genes involved in protein synthesis are again the most numerous (15% of all 2197 genes) and the most highly expressed (25% of overall signal intensity). The increase of other over-represented functional categories (metabolism, protein fate) was less dramatic with only a 1–3% increase in gene number and signal intensity. A different figure was obtained when only genes specific to the male gametophyte were analysed (423 genes). Highly expressed, but mostly constitutive genes involved in protein synthesis disappeared (only 2% genes and signal) and were replaced by newly emerging gene categories of cell wall metabolism (massive increase to 6% gene number and 12% signal) and transport (7% genes, 11% signal). Both of these categories were even more dominant among highly expressed genes, together comprising nearly one third of the overall expression signal.

3.4.2 Transcription in Developing Pollen

The transcriptome of mature pollen grains differs markedly from all other tissues and is accompanied by a significant reduction in complexity (Honys and Twell 2003, 2004). Moreover, although the down-regulation of most microspore-expressed genes is a general trend, a significant group of genes

[5] Genes with the expression signal up to tenfold less than the maximum signal.

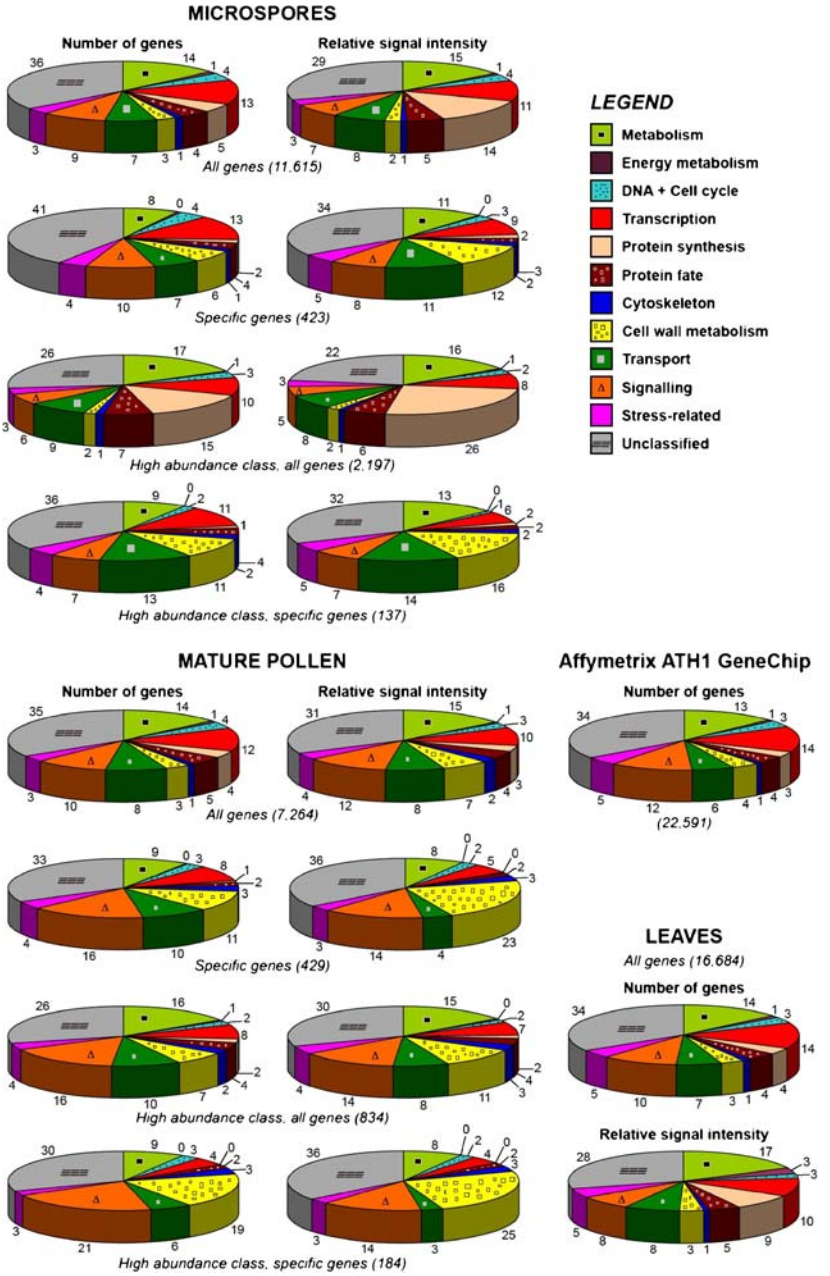

Fig. 5 Distribution of expressed mRNAs among gene function categories. Proportional representation of both the number of genes and their relative expression signals among gene function categories is presented for early (microspore) and late (mature pollen) developmental stages. The equivalent distribution of all genes on the ATH1 array and genes expressed in leaves are shown as controls

are specifically up-regulated late during pollen development. However, this reduction in complexity and parallel activation of different gene sets is not uniform across gene function categories (Fig. 5). Compared to microspores, there was a massive reduction in the protein synthesis group. On the contrary, the most over-represented gene categories were cell wall metabolism, cytoskeleton and signaling. For example genes involved in cell wall synthesis and metabolism comprise more than 19% of highly expressed specific mRNAs (25% signal). Other important functional categories such as transport and stress-related, mostly contained less abundant non-pollen-specific genes. From this perspective, 23 K GeneChip experiments were confirmatory for mature pollen, since these gene function categories were found to be the most massively up-regulated based on analysis of 8K GeneChip data (Honys and Twell 2003).

What was new and surprising was the extent of transcription of mRNAs forming those up-regulated gene function categories. Since we knew that the majority of the most abundant pollen-expressed mRNAs were also pollen-specific (Fig. 4), they represent a relatively narrow set of genes. Moreover, there was an enormous increase in the average signal per gene in these categories. In sporophyte and early male gametophyte, the average signal/gene was within the range 300–500, whereas in late male gametophyte, this value increased to approximately 1000. With the exception of the protein synthesis category all other gene function groups were more abundantly represented among pollen-specific mRNAs. In particular, the average signal/gene for pollen-specific genes involved in cell wall metabolism reached nearly 6000. All these findings unequivocally confirm the bias of male gametophytic gene expression towards functional specialization required for the proceeding progamic phase involving storage, signaling and rapid pollen tube growth.

3.4.3 Post-transcriptional Regulation

Pollen germination in many species has been shown to be largely independent of transcription but vitally dependent on translation (see Twell 1994, 2002). *Arabidopsis* was shown to follow this general trend (Honys and Twell 2004) and there is compelling evidence that many mRNAs are stored in preparation for translation during tube growth (reviewed in Twell 2002). The late accumulation of pollen mRNAs and the presence of full-length transcripts in pollen tubes demonstrate that many mRNAs survive pollen dehydration and rehydration, some of which are stored as unique mRNP particles, EPPs (Honys et al. 2000).

Developmental transcriptomic studies prove the existence of a large number of stored mRNAs in mature pollen. Most abundant late pollen-expressed transcripts up-regulated after pollen mitosis II fall into this category and represent a stored mRNA charge. These studies also revealed that it is not only

mRNA that are stored but also the pre-formed protein synthesis machinery. Most protein synthesis genes are down-regulated in mature pollen and *Arabidopsis* pollen tubes are strictly dependent on ongoing translation (Honys and Twell 2004).

Transcriptomes and proteomes often differ and the absence of certain genes in transcriptomic data does not necessarily mean the absence of their protein products; transcriptomic data also demand experimental proof. For example a recent hypothesis of inactivation of the small RNA pathway in late male gametophyte (Pina et al. 2005) already deserves revision. This hypothesis was formulated on the absence of mRNAs encoding proteins involved in the small RNA pathway in mature pollen. Those mRNAs were found to be present earlier in male gametophyte development and to follow the general trend of down-regulation (Honys and Twell 2004). Our re-analysis[4] of all available mature pollen datasets revealed that one gene (At2g32940) was expressed in Landsberg pollen (Honys and Twell 2004), two genes (At1g48410, At2g27040) were active in the AtGE73 dataset from Columbia pollen (Zimmermann et al. 2004) and At5g21150 was expressed in all three datasets. Surprisingly, application of the normalisation algorithm[6] used by Pina et al. (2005) still identified three of these genes (At1g48410 (Argonaute 1), At2g32940 At5g21150) as active in the AtGE73 datasets from the same ecotype (Zimmermann et al. 2004). Such inconsistencies illustrate the importance of inclusion of all publicly available datasets in such analyses. Moreover, there are examples of the successful application of RNAi approaches in the male gametophyte (Gupta et al. 2002; S-A. Oh, M. Das, J.A. Johnson and D. Twell unpublished results). Taken together, available experimental and developmental transcriptomic data favor the function of the small RNA pathway at earlier stages in development and its persistent activity analogous to the presynthesis and utilization of the protein synthesis machinery in pollen tubes.

3.5 Conclusions from Transcriptomic Studies

Pollen transcriptomic studies provide the first comprehensive genome-wide view of the complexity of gene expression and its dynamics during single cell development in plants. Male gametogenesis is accompanied by large-scale repression of gene expression associated with the termination of cell proliferation and the selective activation of new groups of genes involved in maturation and post-pollination events. Development is also associated with major early and late transcriptional programs and the expression of around 600 putative transcription factors as potential regulators. These data

[6] MAS5 detection algorithm, dChip 1.3 software (http://www.dchip.org; Li and Wong 2001), inclusive treatment of replicates for identification of expressed genes

also highlight a diminished role for transcription and the important role of mRNA and protein storage in mature pollen. Over representation of genes involved in cell wall metabolism, cytoskeleton and signaling highlight the functional specialization of pollen in preparation for key progamic functions including recognition of target tissues and rapid directional pollen tube growth.

4 Perspectives – Integrating Genetic and Transcriptomic Data

Genetic screens for male gametophytic mutations are far from reaching saturation given that almost all mutations are represented by single alleles. Estimates of the number of genes that are essential for male gametophyte development including the progamic phase can be based on equivalent segregation ratio distortion screens in which all mutants affecting transmission were characterized (Lalanne et al. 2004; Johnson et al. 2004). Based on the assumption that approximately 180 000 T-DNA insertions are required to achieve saturation, Johnson et al. (2004) estimated that ~ 330 genes would be identified. Similar calculations for the transposon-based Ds screen (Lalanne et al. 2004) would predict ~ 575 genes. However the proportion of mutants affecting development in these two screens varied from 10% (T-DNA) to 30% (Ds) corresponding to ~ 30 to 170 genes respectively. In stark contrast, estimates of the total number of genes expressed throughout male gametophyte development based on microarray data are ~ 11 000 to 14 000 (Honys and Twell 2004). Moreover, because of the frequent co-expression of gene family members in developing pollen (Honys and Twell 2004), genetic redundancy is likely to be a major feature and will be a significant limitation to genetic screens. To combat this deficiency, comprehensive transcriptome data can now be used to accelerate and target functional studies using reverse genetics. Targeted genetic approaches include assembly of selected multiple knockout mutants based on co-expression data. An alternative solution for closely related genes likely to be redundant is to down regulate multiple family members using antisense or RNAi approaches. This will be made more effective if cell-and stage-specific promoters can be identified based on microarray expression profiles. Promoters that are specifically active in generative and sperm cells and at late stages in pollen development have now been identified (Engel et al. 2005; Rotman et al. 2005), and microarray data analysis has enabled the isolation of promoters specifically active at microspore stage (D. Honys, S-A Oh, D. Twell, unpublished). In conclusion, the integration of established genetic approaches and resources with recent trancriptomic data analysis heralds an exciting new era in which the comprehensive identification of male gametophytic gene functions is a realistic goal.

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Ions and Pollen Tube Growth

Peter K. Hepler[1] (✉) · Alenka Lovy-Wheeler[1] · Sylvester T. McKenna[2] · Joseph G. Kunkel[1]

[1]Department of Biology, University of Massachusetts, Amherst, MA 01003, USA
hepler@bio.umass.edu

[2]Department of Biology, Long Island University, Brooklyn, NY 11201, USA

Abstract Ions play a crucial role in the control of pollen tube growth. In this review we focus on four that seem especially important: calcium (Ca^{2+}), protons (H^+), potassium (K^+), and chloride (Cl^-). Ca^{2+} in the extracellular medium is essential for growth; it forms a steep intracellular tip-focused gradient, and exhibits a prominent extracellular tip-directed Ca^{2+} influx. pH is also essential for growth. H^+ form an intracellular gradient consisting of a slightly acidic domain at the extreme apex and an alkaline band located along the clear zone. H^+ also exhibit an apical influx, but in contrast to Ca^{2+} show an efflux along the clear zone, in the region occupied by the intracellular alkaline band. K^+ and anions (possibly Cl^-) appear to participate in the growth process, as evidenced by the striking extracellular fluxes that are associated with tube elongation. K^+ exhibits an apical influx, while an anion displays an apical efflux. An exciting finding has been the discovery that pollen tube growth oscillates in rate, as do all the ionic expressions noted above. While the ionic activities and fluxes show the same period as growth, they usually do not show the same phase. The exploration of phase relationships, using cross-correlation analysis, reveals that most ion expressions lag growth. Thus, intracellular Ca^{2+} activity follows growth rate by 1–4 s, whereas extracellular Ca^{2+} influx follows growth rate by 12–15 s (130°). These observations suggest that Ca^{2+} is a follower rather than a leader in growth. Despite the knowledge that has been gained, several aspects of ionic expression and function remain to be determined. Their elucidation will contribute greatly to our overall understanding of the control of pollen tube growth.

1 Introduction

It is well known that ions play a central role in the control of pollen tube growth. Over 40 years ago Brewbaker and Kwack (1963) revealed that in vitro culture of pollen tubes required calcium (Ca^{2+}). This initial finding prompted considerable further work on Ca^{2+}, with the establishment of the necessary limits (10 μM to 10 mM) (Steer and Steer 1989), the discovery of local gradients and fluxes, and the characterization of numerous targets in both the cytoplasm and cell wall through which this ion can influence pollen tube growth. But Ca^{2+} is not the only ion that directly participates in pollen tube growth; there are others including notably protons (H^+), potassium (K^+),

and possibly chloride (Cl^-) that contribute to the growth process (Holdaway-Clarke and Hepler 2003).

As we ponder the ways in which ions contribute to growth it becomes immediately apparent that many processes are involved. For example, turgor pressure, which drives growth, is dependent on the regulation of certain ions, especially K^+ and anions (possibly Cl^-). H^+ participate in many fundamental processes; perhaps most central are the transmembrane pH gradients, driven by the H^+-ATPase that contribute to energetic and membrane transport processes. Ca^{2+}, widely recognized as a mediator in signal transduction for all eukaryotic cells, has no less of a role in pollen tubes. Potential targets include cell motility and the cytoskeleton, exo- and endocytosis, and cell wall structure, all of which are central to the growth of the pollen tube (see the chapters by Malhó, Yokota and Shimmen, and Geitmann and Steer, this volume).

Our understanding of ions has improved enormously as a result of key technical developments. The use of intracellular reporters has allowed us to observe both temporally and spatially the intracellular activities of these ions during the pollen tube growth. Secondly, the development of extracellular probes, especially those that possess ion selectivity (Kühtreiber and Jaffe 1990), has allowed us to detect the location, direction, and magnitude of extracellular ion fluxes for Ca^{2+}, H^+, K^+, and Cl^-. Our goal in this chapter is to provide an overview of what is known about the relationship between ions and pollen tube growth, and attempt to place this knowledge within a wider context.

2
Ion Gradients and Fluxes

2.1
Ca^{2+}

An exciting and compelling result that emerges from the use of ratiometric indicator dyes is the presence of a striking "tip-focused" gradient in Ca^{2+} activity that is located in the apical domain of the growing pollen tube, immediately adjacent to the region of maximal elongation (Rathore et al. 1991; Miller et al. 1992) (Fig. 1a). Studies with fura-2-dextran indicate that the gradient extends from $\sim 3\ \mu M$ at the extreme apex of the tube to a basal level of $0.17\ \mu M$ within $20\ \mu m$ of the apex (Pierson et al. 1994, 1996). Because of its pK_a for Ca^{2+}, which is $0.57\ \mu M$, fura-2-dextran is close to saturation at $3\ \mu M$, and as a consequence the dye may under-report the true apical $[Ca^{2+}]$. Results using the Ca^{2+}-sensitive photoprotein, aequorin, which is capable of reporting elevated $[Ca^{2+}]$, indicate that the concentration at the apex may be as high as $10\ \mu M$ (Messerli et al. 2000). An important recent advance has been the introduction of cameleon, a transfectable Ca^{2+} indicator that allows observa-

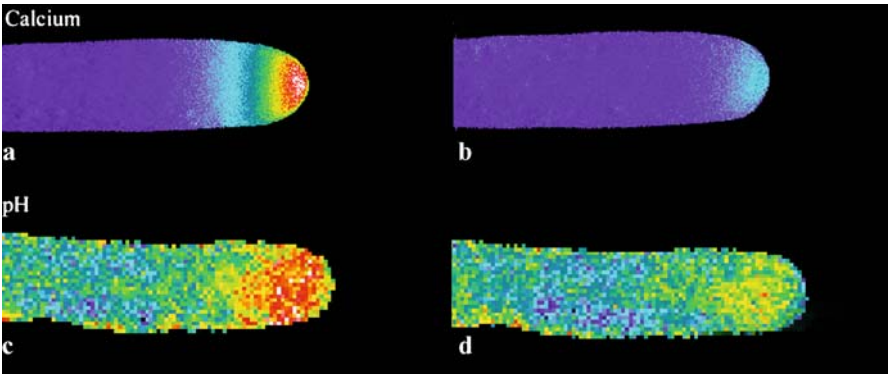

Fig. 1 Calcium and pH. **a**,**b** Changes in magnitude of the tip-focused Ca^{2+} gradient during oscillatory growth. The cytosolic [Ca^{2+}] oscillates between high (**a**) and low (**b**) levels. **c**,**d** Changes in magnitude of the apical pH gradient, which oscillates between high (**c**) and low (**d**) levels

tion of the gradient in pollen tubes including those of *Arabidopsis* growing in vivo (Iwano et al. 2004; Watahiki et al. 2004). The tip-focused gradient can be viewed as a standing wave at the apex which carries important information. Thus Ca^{2+}-responsive proteins, such as calmodulin, will be saturated (and active?) at the extreme apex, whereas these same factors will lack this ion just 20 μm away from the tip and be inactive. Within this framework it is easy to envision how the Ca^{2+} gradient spatially regulates a host of activities at the apex of the pollen tube.

The extracellular flux of Ca^{2+} in many ways mirrors the intracellular gradient. Studies with a Ca^{2+}-selective vibrating electrode reveal an extracellular influx of Ca^{2+} that is focused toward the tip of the tube (Kühtreiber and Jaffe 1990) and is of substantial magnitude (20 pmol/cm^2/s) (Holdaway-Clarke et al. 1997). No efflux of Ca^{2+} has been observed. Both the apical influx of extracellular Ca^{2+} and the expression of the intracellular tip-focused gradient are dissipated by several experimental conditions that inhibit pollen tube elongation. Injection of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) Ca^{2+} buffers simultaneously reduces the gradient, blocks extracellular influx, and inhibits tube elongation (Pierson et al. 1994). But other agents, some of which do not seem related to either intracellular or extracellular Ca^{2+}, achieve the same effects. Thus, inhibition of growth with increased osmoticum, application of a mild thermal shock, or incubation in caffeine, all similarly reduce the tip-focused gradient and eliminate the extracellular influx (Pierson et al. 1996). These effects can be reversed by a return to normal growth conditions with the reappearance of growth together with the reemergence of the intracellular gradient and the extracellular influx. Taken together these results suggest that there is a close coupling between the intracellular gradient and extracellular influx.

A question of ongoing interest concerns the source of Ca^{2+}. The simplest explanation is that the gradient derives directly from the influx of extracellular Ca^{2+} (Fig. 2). This view is supported by studies in which Mn^{2+}, added to the medium, quenched the indicator dye fluorescence; presumably Mn^{2+} gained access to the cytoplasm by passing through Ca^{2+} channels (Malhó et al. 1995). It has long seemed plausible that the deformation of the plasma membrane at the tip, which presumably occurs during turgor-dependent cell elongation, would be sufficient to open mechanosensitive Ca^{2+} channels, allowing the rapid influx of this ion down its electrochemical gradient. Using patch-clamp electrophysiology, Dutta and Robinson (2004) have identified the postulated stretch-activated Ca^{2+} channels; these are associated with the plasma membrane on both the grain and tip of lily pollen tubes. In studies on the grain, the membrane containing the stretch-activated Ca^{2+} channels came from the region where the tube will emerge during germination. To access the apical membrane on pollen tubes, cells were first plasmolyzed, then the apical wall was digested with pectinase, and finally the plasmolysis conditions were reversed, which led to the extrusion of a protoplast that could be subjected to patch-clamp analysis (Dutta and Robinson 2004). Here, they detected a stretch-activated Ca^{2+} channel (conductance ~ 15 pS), similar in properties to those on the grain. Of further pertinence, these channels, as well as elongation of the pollen tube, could be blocked by application of crude spi-

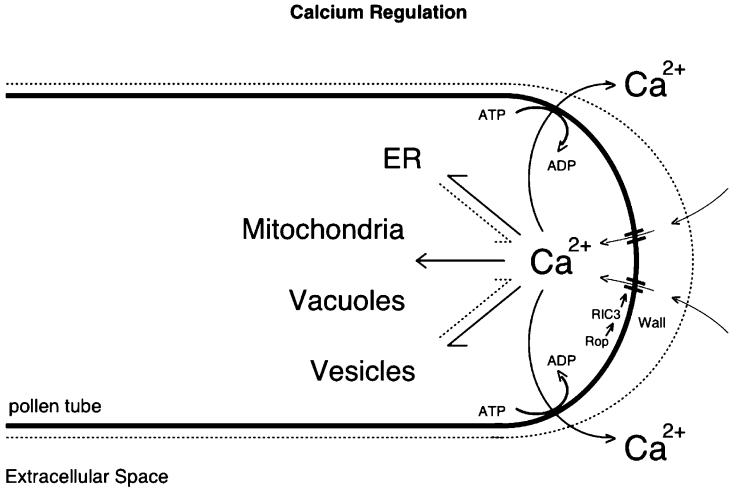

Fig. 2 Likely pathways for the regulation of cytosolic Ca^{2+} in the growing pollen tube. The influx is shown as an *interrupted line* suggesting that Ca^{2+} interacts with cell wall components. Extrusion and sequestration of the ion from the cytosol is presumably accomplished by pumps on the plasma membrane (see the chapter by Sze et al., this volume), the ER, vacuole, mitochondria, and vesicles (*solid lines*). *Dashed lines* represent postulated pathways from cytoplasmic organelles, such as the ER and vesicles, toward the Ca^{2+} gradient

der venom, previously reported to block stretch-activated channels in other membranes (Dutta and Robinson 2004).

While the participation of an extracellular influx seems likely, other sources of Ca^{2+} may contribute to the gradient. Given the presence of the endoplasmic reticulum (ER) and especially the inverted cone of secretory vesicles in the apical domain (Lancelle and Hepler 1992), it is possible that release from these stores could contribute to the intracellular gradient (Fig. 2). Inositol 1,4,5-trisphosphate (IP_3) has been shown to cause an increase in the intracellular [Ca^{2+}] (Franklin-Tong et al. 1996; Malhó 1998), but whether or not this is a natural regulator still requires experimental verification. On balance, the position of the gradient with its maximal level immediately adjacent to the plasma membrane, together with new evidence showing the presence of stretch-activated Ca^{2+} channels in the apical membrane, argue persuasively for the idea that Ca^{2+} influx from the extracellular space is the primary source of this ion during pollen tube growth.

Although we cannot be certain that Ca^{2+} release occurs from the ER and other organelles, it is likely that uptake of Ca^{2+} by these intracellular components, and/or the extrusion of Ca^{2+} at the plasma membrane, play a major role in governing the profile and extent of the tip-focused gradient (Fig. 2). It is generally well appreciated that Ca^{2+} pumps on the plasma membrane, ER, mitochondria, and central vacuole actively participate in the removal of excess Ca^{2+} from the cytosol (Sze et al. 2000). Exactly how these various uptake processes contribute to the conditions observed in the pollen tube is not known in detail, but recent studies provide evidence for a Ca^{2+}-ATPase (ACA9) that is required for pollen tube growth and for proper fertilization (Schiøtt et al. 2004). ACA9, which is a member of the family of autoinhibited Ca^{2+}-ATPases, is expressed predominantly in the male gametophytic tissue, and in pollen tubes is located on the plasma membrane (Fig. 2; chapter by Sze et al., this volume).

A further important issue to consider in our attempts to understand Ca^{2+} regulation concerns the contribution of the cell wall. While the cytoplasmic indicator dyes specifically report on ion activities within that compartment, the identity of the compartment that influences the signal from the Ca^{2+}-selective vibrating electrode is less clear. Although it has been assumed that the signal is due to movement of ions across the plasma membrane (Speksnijder et al. 1989), it must be kept in mind that influx of Ca^{2+} from the extracellular medium must first cross the cell wall. If there is any binding to cell wall components this will contribute to the signal. Since the cell wall at the apex is composed of pectins, which following de-esterification contain carboxyl residues, there are numerous potential Ca^{2+} binding sites. Indeed, calculations suggest that the Ca^{2+}–cell wall interaction dominates the apparent extracellular influx of Ca^{2+}, with the number of ions crossing the plasma membrane at the apex being less than 10% of the total signal (Holdaway-Clarke and Hepler, 2003). A Ca^{2+}–cell wall interaction is thus important; it is

a major activity that must be considered in any model of pollen tube growth and its control.

2.2 H^+

In addition to Ca^{2+}, pollen tube growth is regulated by pH (Fricker et al. 1997; Feijó et al. 1999). Acidic conditions are necessary, with pH 7 being unable to support tube elongation (Holdaway-Clarke et al. 2003). Studies on the intracellular distribution of H^+ reveal that pollen tubes possess a unique intracellular pH gradient (Feijó et al. 1999). However, because of the much greater mobility of H^+ when compared to that of Ca^{2+}, it has been difficult to observe these pH gradients (Fricker et al. 1997; Parton et al. 1997; Messerli and Robinson 1998). The indicator dyes, especially when used at more elevated levels, appear to locally buffer the pollen tube cytoplasm and dissipate the activity that is being sought. When low concentrations of the indicator BCECF–dextran are used (0.3–0.5 μM) a gradient in pH becomes evident, which consists of a slightly acidic (pH = 6.8) apex, with an alkaline band (pH = 7.5) toward the base of the clear zone (Feijó et al. 1999) (Fig. 1c). We presume that the alkaline band is confined to the cell cortex, where it is governed by plasma membrane H^+-ATPases (see the chapter by Sze et al., this volume). In parallel with these intracellular studies, experiments with a H^+-selective vibrating electrode indicate an extracellular influx at the apex of the tube, with a distinct efflux at the edge of the cell near the clear zone. Thus, in marked contrast to Ca^{2+}, the flux pattern for H^+ reveals a current loop, which might be effective as a polarizing factor, especially in the confined spaces of the pistil during fertilization in vivo.

Inhibition of pollen tube growth eliminates the acidic tip but the alkaline band persists, and even extends more closely to the apex (Feijó et al. 1999). These observations are consistent with the influx of H^+ at the tip being dependent on growth and the attendant deformation of the apical plasma membrane. Indeed, it is plausible that H^+ enter through the same stretch-activated channel as Ca^{2+}, thus explaining the marked dependence on growth for the entry of both of these ions. The alkaline band, by contrast, presumably derives from the activity of a plasma membrane H^+-ATPase, which throughout plants is recognized as a key regulatory enzyme for energizing transport processes (Palmgren 2001; chapter by Sze et al., this volume). It is pertinent that inhibitors of the H^+-ATPase, including vanadate, azide, and *N*-ethylmaleimide, block pollen tube growth, while its stimulation by fusicoccin enhances growth (Rodriguez-Rosales et al. 1989; Feijó et al. 1992; Fricker et al. 1997; Pertl et al. 2001). Cytological evidence for a plasma membrane ATPase has thus far been mixed. Feijó et al. (1992) showed ATPase activity on the plasma membrane of both the grain and pollen tube, whereas Obermeyer et al. (1992) only demonstrated strong activity in association with the

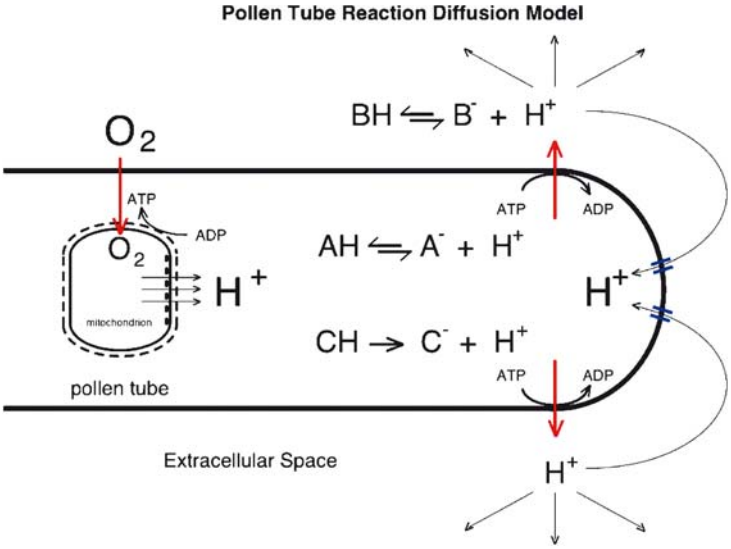

Fig. 3 A nonlinear, reaction–diffusion model for pH regulation [adapted from a model first published by Feijó et al. (1999)]. A principal component is a plasma membrane ATPase that pumps H^+ into the cell wall space leaving behind a domain of OH^-. Mitochondria play a pivotal role as a prime source of ATP; they also generate NAD(P)H and H^+. An important feature of this model is the current loop, in which H^+, extruded by the H^+-ATPase, enter the tip possibly through growth-dependent cation channels. This current loop may serve an important role in defining and controlling pollen tube growth polarity

grain. However, the marked H^+ efflux observed along the clear zone strongly suggests the presence of a H^+-ATPase.

H^+ emerge as potential key regulators of pollen tube growth and polarity (Fig. 3). In an attempt to explain pH regulation, Feijó et al. (1999) put forth a reaction–diffusion model, which emphasizes the contribution of several important factors in the control of local H^+ activity, including intra- and extracellular metabolic reactions, the cytosolic buffering capacity, and the small spatial separation of influx and efflux. When these nonlinear conditions are factored together, a model emerges that supports a stable spatial pattern (Fig. 3) that belongs to a family of processes described by Turing (1952). Simultaneous H^+ and O_2-electrode measurements concur that a key energy insertion point in this model would be the region basal to the inverted cone and alkaline zone that is rich in mitochondria (Kunkel et al. 2005). This area would be a source of both ATP and H^+ which are needed to feed the process. H^+ continue to be dissipated as they are pumped out of the pollen tube's alkaline zone, which in the model is rich in the H^+-ATPase responsible for ejecting H^+. This H^+ ejection feeds a local external low pH, which in turn provides the protons that rhythmically enter at the pollen tube tip

(Fig. 3). Dissipation of the H^+ depends on their order of magnitude higher diffusion coefficient compared to those of other ions as well as the local buffer capacity (Kunkel et al. 2001). The self-regulatory nature of the pollen tube oscillating system was exhibited using phase state attractor diagrams of pollen tube oscillations that once perturbed return to their earlier attractor behavior (Feijó et al. 2001). This modeling approach, which has yet to be fully understood in its application to pollen tube physiology, illustrates the need for novel ways of viewing the complex nonlinear processes that underlie pollen tube tip growth.

2.3 K^+

Early studies on total currents associated with growing pollen tubes came to the conclusion that K^+ was a key ion involved in carrying the observed current (Weisenseel and Jaffe 1976). Furthermore, K^+ entered the apex and exited through the base of the shank or the grain. This question has been readdressed more recently using K^+-selective electrodes. Unfortunately the K^+-selective electrodes often exhibit poor performance (Messerli et al. 1999). The K^+-selective liquid ion exchanger (LIX), which differs in its vehicle from most others, requires a differently shaped and better silanized glass micropipette filled with a longer column of LIX. Nevertheless, with suitable electrodes, it has been possible to document a prominent K^+ influx at the apex of growing pollen tubes.

Considerable evidence exists for the presence of K^+ channels associated with pollen grain or tube plasma membranes. Three different K^+ channels have been detected in lily pollen protoplasts, with the most common exhibiting a conductance of 19 pS (Obermeyer and Kolb 1993; Obermeyer and Blatt 1995). Also, using pollen protoplasts, an inward K^+ channel in *Brassica* has been identified by patch-clamp analysis that is increased in its activity by extracellular Ca^{2+} (10–50 mM), and by an acidic extracellular pH (4.5) (Fan et al. 1999, 2001). By contrast, an outward K^+ channel has been reported that responds to an acidic internal pH (Fan et al. 2003). An inward K^+ channel has also been identified in *Arabidopsis*, both in plasma membranes from the grain and from the apical region of the tube (Mouline et al. 2002). Its conductance of 14 pS suggests that this channel from *Arabidopsis* may be similar to the 19-pS K^+ channel found in lily (Obermeyer and Kolb 1993). It is additionally important to note that a mutation in the *Arabidopsis* K^+ channel caused reduced ion uptake and correspondingly reduced growth (Mouline et al. 2002). Finally, both stretch-activated and spontaneous K^+ channels have been identified by patch-clamp analysis (Dutta and Robinson 2004). The stretch-activated channels are observed in the groove on the protoplast derived from the pollen grain, whereas the spontaneous K^+ channel occurs over the whole surface. Curiously, neither channel was detected in

membranes derived from pollen tube protoplasts. Like the stretch-activated Ca^{2+} channel, the stretch-activated K^+ channel, but not the spontaneous K^+ channel, is inhibited by spider venom (Dutta and Robinson 2004).

2.4 Cl^-

The data and conclusions concerning the participation of K^+ in pollen tube growth are unambiguous, whereas the status of Cl^- is less clear. Zonia et al. (2002) reported a marked efflux of this ion from the apex of tobacco pollen tubes, with an influx occurring along the flanks of the tube starting 12 μm back from the tip. Thus, like H^+, Cl^- fluxes form a current loop in the apical domain, and may play a key role in growth polarity. The inhibition of tube growth together with the initiation of apical swelling through the application of IP_4, a putative Cl^- channel blocker, are consistent with the conclusion that transport of this anion is crucial to pollen tube growth, where presumably it would play a role in turgor regulation. However, these results have been contested by Messerli et al. (2004) who assert that the LIX used by Zonia et al. (2002) is not specific for Cl^-. They further question the idea that Cl^- channels or transport are necessary for pollen tube growth and survival.

The cautions of Messerli et al. (2004) are to be taken seriously. No LIX used for ion-probe measurement is absolutely specific, and there are many instances of interactions of LIX within electrodes with the media in which they are operated. The published relative affinities of a given LIX for related ions were determined for static macroelectrodes and are often dramatically different from the relative affinities measured with microelectrodes, as demonstrated by Messerli et al. (2004). In the case of the Cl^--LIX, Messerli et al. (2004) reported that nitrate provides a substantial relative signal. This type of interference in which the LIX itself carries another ion is a serious conflict that must be overcome. A more serious assertion about the Cl^--LIX is that it reacts with 2-(*N*-morpholino)ethanesulfonic acid (MES), the zwitterionic buffer (Good et al. 1966) commonly used in pollen tube studies because of its favorable pK. MES has a different interaction with the LIX in its protonated vs unprotonated form that potentially makes the LIX a proton sensor in the presence of MES as well as a Cl^- electrode. In the given example, in which Zonia et al. (2002) measure the efflux of Cl^- from the tip of the pollen tube, it is asserted that they are actually measuring the previously reported oscillating proton current (Messerli et al. 2004).

This discrepancy in the interpretation of data may have technical reasons. Messerli et al. (2004) examined the responsiveness of static electrodes to ions using direct-coupled potentiometers, which provide more accurate estimates of relative sensitivities to different ions (including a tenfold greater sensitivity to nitrate and a greater sensitivity of the protonated form of the buffer MES). The direct-coupled potentiometers are reported to give a rapid rise in

the response over a negligible time interval (0.25 ms). However, the voltage measurement in live cells was not done with the direct-coupled potentiometer but rather with self-referencing capacitative electronics, which are not as stable as direct-coupled electrodes. This results in less-linear calibrations and low, voltage-dependent efficiencies [85% efficiency in direct-coupled oscillating mode (Kunkel et al. 2005)]. As a result the conclusions of Messerli et al. (2004) must be examined with caution. Clearly, further experiments in a buffer that does not interfere with the Cl^--LIX are required before we can draw conclusions about the role of Cl^- and other anions in tip growth.

3
Oscillations in Pollen Tube Growth and Associated Ion Expression

An important finding has been the discovery that the rate of pollen tube growth oscillates (Pierson et al. 1995). In vitro, lily pollen tubes longer than 600–700 μm exhibit a change in growth rate from 100 to 500 nm/s over a period of 15–50 s (Pierson et al. 1996). Additionally, the intracellular activities of both Ca^{2+} and H^+, as well as the extracellular fluxes of Ca^{2+}, H^+, K^+, and Cl^-, all exhibited oscillatory profiles which possessed the same period as that of growth, but usually not the same phase (Holdaway-Clarke and Hepler 2003). These observations allow us to decipher the phase relationship between an ion activity or its extracellular flux and the underlying rate of growth. Further, by determining whether an ion expression precedes or follows growth, information can be gained about those events or processes that anticipate and possibly regulate growth, as opposed to those that follow and appear to be governed by the preceding growth event.

When two processes oscillate, but not with the same phase, it is not immediately obvious which one precedes the other. To solve this problem, cross-correlation analysis has been used. Cross-correlation analysis of simultaneously collected time series processes, e.g., tip growth and process "*x*", allows the strength of correlation and the lag between two processes to be established (Brillinger 1981).

3.1
Ca^{2+} Oscillations

Examination of the intracellular $[Ca^{2+}]$ during oscillatory growth indicates that the changes, like those in the growth rate, are substantial, with values from 750 to 3500 nM being observed (Pierson et al. 1996) (Fig. 1a,b). Although initial inspection suggested that intracellular Ca^{2+} and growth were in phase (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997), further studies at higher temporal resolution revealed surprisingly that the maximum $[Ca^{2+}]$ in the tip-focused gradient peaked 1–4 s, or ~ 20°, after the peak in

growth rate (Messerli et al. 2000). These observations indicate that Ca^{2+} activity is not a leader of growth but a follower; its changes are dictated by growth rate changes, rather than vice versa. Of further interest, the extracellular influx of Ca^{2+}, which also oscillates, is delayed in phase from the intracellular Ca^{2+} activity. Thus Ca^{2+} influx lags growth by 135°, and intracellular Ca^{2+} activity by 115° (Holdaway-Clarke et al. 1997). These data put constraints on the popular idea that Ca^{2+} is regulating the process of growth; rather it would appear that the growth process determines the subsequent expression of both the intracellular gradient and the extracellular influx.

A further outcome of these data has been the necessity to rethink the relationship between the extracellular Ca^{2+} influx and the intracellular gradient. The influx measurements are a composite that unavoidably includes information about Ca^{2+} entry into the cell wall domain, as well as Ca^{2+} entry into the cytoplasm (Holdaway-Clarke et al. 1997). However, there is not a full consensus on this issue, as others believe that intracellular stores, such as the ER or the secretory vesicles, take up significant amounts of Ca^{2+} and thereby may account for the large influx signal (Malhó and Trewavas 1996; Messerli and Robinson 2003). Whatever the mechanism, it is clear that some form of Ca^{2+} storage is required to account for the marked phase separation in the expression of the intracellular Ca^{2+} gradient and the influx of extracellular Ca^{2+}.

3.2
H^+ Oscillations

In the initial studies on H^+ imaging in pollen tubes it was noted that the intracellular pH oscillates, with the alkaline band being out of phase with the growth rate (Feijó et al. 1999) (Fig. 1c,d). Due to low signal levels, and the tendency for the dye to bleach following rapid sequential measurements, it was not possible at the time to gain the necessary temporal resolution that would allow the definitive measurement of the phase relationship. This question is currently under investigation using more sensitive equipment. Preliminary results indicate that formation of the alkaline band peaks before growth by 3–7 s (~ 45°), whereas the acidic tip follows growth by 3–6 s (~ 40°) (Hepler et al. 2005; Lovy-Wheeler et al. 2005a). These findings point to H^+ pumping and the generation of the alkaline band as an anticipatory event that may serve as a key regulatory event in the control of cell growth.

The extracellular influx of H^+ oscillates, but thus far an oscillatory efflux along the sides of the clear zone, close to the intracellular alkaline band, has not been reported, despite the observation that the alkaline band oscillates. The oscillatory apical influx, when subjected to cross-correlation analysis, lags growth by ~ 100°, and consequently is out of phase with the oscillation of the intracellular acidic domain at the pollen tube tip (Messerli et al. 1999). Again, cell wall generation or binding of H^+ might explain these seeming dis-

continuities. For example, when methylated pectins are de-esterified, H^+ are released. Also H^+ fluxes will be associated with the binding of cations to cell wall components.

3.3
K^+ and Cl^- Oscillations

Thus far we do not have information concerning the oscillations, if any, in the intracellular activities of these ions, but the extracellular fluxes of both ions oscillate in relation to the changes in growth rate. The extracellular influx of K^+ presents a very similar phase relationship to growth as do H^+, and is also not significantly different from that of Ca^{2+}; thus, the influx of K^+ lags growth by about 100° (Messerli et al. 1999). Cl^-, in contrast to these other ions, exhibits a marked efflux from the apex, and an influx along the sides of the clear zone (Zonia et al. 2002). Similarly to H^+, only the activity at the apex has been observed to oscillate. In tobacco pollen tubes, Cl^- efflux occurs in exact phase with the growth rate (Zonia et al. 2002). However, as noted earlier, these results have been challenged by Messerli et al. (2004) raising the hypothesis that it may be another anion or H^+ that are being measured by Zonia et al. (2002).

4
Targets for Ion Action

4.1
Cytoskeleton and Motile Processes

The cytoskeleton, especially that composed of actin, plays a crucial role in the control of pollen tube growth. Actomyosin is responsible for driving cytoplasmic streaming, and the transport of the secretory vesicles to the apical domain. But separate from its role in streaming, actin polymerization/turnover in the apical domain is required for pollen tube growth (Gibbon et al. 1999; Vidali et al. 2001; chapter by Yokota and Shimmen, this volume). Thus, agents that block polymerization or turnover, including profilin, DNAse, latrunculin-B, and cytochalasin-D, all inhibit cell elongation at a significantly lower concentration than that needed to stop cytoplasmic streaming (Vidali et al. 2001). Although there has been controversy over the structure of actin, especially in the apical domain, the recent work of Lovy-Wheeler et al. (2005b), using rapid freeze fixation coupled with antibody labeling, provides high-quality images of the actin cytoskeleton in fixed cells. The results revealed the presence of a prominent collar or cortical fringe of F-actin in the apical domain (see the chapter by Yokota and Shimmen, this volume). Starting 1–5 μm back from the tip, this system of longitudinally aligned filaments extends basally through the cell cortex for another 5–10 μm. Thereafter, actin

microfilaments are finely articulated and evenly dispersed throughout the thickness of the tube and extend throughout the shank.

Since ions, especially Ca^{2+} but also H^+, can modulate the actin cytoskeleton in other systems, it seems likely they will do so in pollen tubes. It has been known for years that injection of Ca^{2+} into pollen tubes causes fragmentation of F-actin (Kohno and Shimmen 1987). More recently, different actin binding proteins have been identified, notably myosin, profilin, villin/gelsolin, and ADF, which in response to Ca^{2+} or H^+ exhibit an altered activity toward actin to control its structure or activity. These interactions are described in detail in the chapter by Yokota and Shimmen (this volume) and in Fig. 4. Here we introduce only a few comments on their spatial location and significance for pollen tube growth. Firstly, the tip-focused Ca^{2+} gradient, acting together with profilin and villin/gelsolin, will prevent polymerization and/or fragment existing microfilaments. Together they account for the marked reduction of F-actin in the extreme apex of the pollen tube. In addition, the high $[Ca^{2+}]$, which is sufficient to inhibit myosin, can explain the absence of cytoplasmic streaming in this extreme apical domain. Secondly, the alkaline band can be expected to activate ADF, a pH-sensitive actin binding protein (Allwood et al. 2002; Chen et al. 2002). The colocalization of the alkaline band with the actin fringe invites speculation about a functional interaction. Spe-

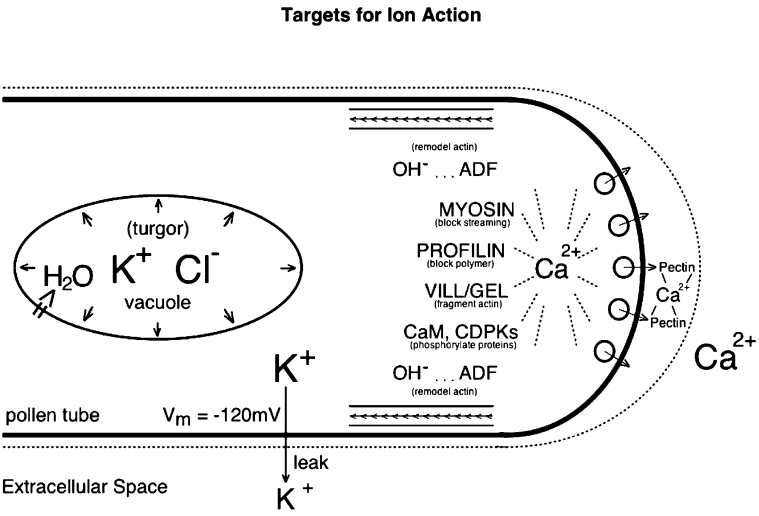

Fig. 4 Several targets and associated pathways for ion-modulated events that appear to play a crucial role in the control of pollen tube growth. The tip-focused Ca^{2+} gradient stimulates secretion, affects the structure and activity of the actin cytoskeleton, and regulates protein phosphorylation. The alkaline band is depicted as specifically modulating the cortical actin fringe through stimulation of actin remodeling by ADF. K^+ and anion (possibly Cl^-) accumulation in the vacuole, together with water uptake, regulate turgor pressure. An outward leakage of K^+, coupled with the retention of fixed negative charges, controls the membrane potential

cifically, the alkaline band through the activation of ADF could contribute to the turnover of the cortical fringe, and contribute importantly to growth polarity. These observations resonate closely with recent work from animal systems (Bernstein and Bamburg 2004). Studies on fibroblasts show that cells lacking the Na^+/H^+ antiporter, and unable to generate localized pH gradients, also lack cytoskeletal anchoring and polarity (Denker and Barber 2002). Among the key cytoskeletal proteins, ADF/cofilin emerges as the most likely candidate to control cell polarity (Ghosh et al. 2004). Bernstein and Bamburg (2004) enlarge this relationship into a comprehensive model for the generation of polarity in animal cells. They suggest that local pH changes at the plasma membrane are the key factor in tipping the balance for F-actin remodeling, and thus in defining the origin of polarity.

4.2 Endocytosis/Exocytosis

It is commonly accepted in plant and animal cells that Ca^{2+} facilitates secretion (Battey et al. 1999). Thus, a strong candidate function for the tip-focused Ca^{2+} gradient would be the stimulation of exocytosis of the apically accumulated vesicles, which contain the cell wall precursors needed for cell elongation (Fig. 4; chapter by Malhó, this volume). Experimental probing of this idea shows that elevation of the Ca^{2+} levels, brought about by local uncaging of a light-sensitive Ca^{2+}-containing reagent, stimulates exocytosis, with the latter being inferred from a reduction in FM1-43 fluorescence (Camacho and Malhó 2003). However, in parallel studies, increasing the concentration of GTPγ S, a nonhydrolyzable analog of GTP, increased exocytosis concomitant with a slight decrease in Ca^{2+}. While these results may seem inconsistent with the idea that Ca^{2+} facilitates secretion, it must be realized that the pollen tubes still exhibit an apical gradient, and thus at the point of secretion the local concentration of Ca^{2+} is well above basal levels. Studies in maize coleoptiles have shown that the secretory process saturates above 1.5 μM, with half maximal stimulation at $\sim 0.9\ \mu$M (Sutter et al. 2000). If the pollen tube is similar, then even when the gradient is at its low point ($\sim 0.75\ \mu$M), there is still sufficient Ca^{2+} to stimulate the secretory process (Holdaway-Clarke and Hepler 2003).

The connection between Ca^{2+} and secretion is also supported by studies using Yariv reagent to block pollen tube growth (Roy et al. 1999). Under these conditions, elongation stops, whereas secretion continues with irregular wall thickenings arising, which are accompanied by elevated levels of Ca^{2+} in the adjacent cytoplasm (Roy et al. 1999). It is possible that stretch-activated channels are involved, with the deformation resulting from secretion causing mechanical strain that opens a Ca^{2+} channel. The recent work of Pickard and Fujiki (2005) supports this contention. In cultured BY-2 cells, they show by patch-clamp analysis that inactivation of the wall-associated arabinogalactan

proteins with Yariv reagent deregulates the activity of the stretch-activated Ca^{2+} channels, allowing uncontrolled ion influx. These authors promote the idea of a cortical plasma membrane-associated reticulum, in which a complex of cytoskeleton and arabinogalactan proteins creates a force-focusing system that spatially confines and regulates the activity of stretch-activated channels (Pickard and Fujiki 2005).

4.3 Ion Binding Proteins

A widely accepted function for Ca^{2+} is the ability to activate a process through an intermediary binding protein. Calmodulin (CaM) emerges as a likely candidate protein that can transmit the Ca^{2+} signal to a response element such as a protein kinase (Snedden and Fromm 2001). A second factor is the large family of Ca^{2+}-dependent protein kinases (CDPKs) (Harmon et al. 2001; Harper et al. 2004). These proteins, which are directly regulated by Ca^{2+}, are uniquely found in plants and a few protists, but not in yeast or animal cells.

4.3.1 Calmodulin

Current studies derived from live cells injected with fluorescently labeled calmodulin reveal that this protein is evenly distributed throughout the pollen tube cytoplasm (Moutinho et al. 1998b). Whereas total calmodulin may not accumulate in the apex, that which is activated by binding to Ca^{2+} is elevated in a pattern that is similar to the tip-focused gradient (Rato et al. 2004). These findings have been gained through the use of TA-CaM, a fluorescent analog of calmodulin that changes its quantum yield when bound to Ca^{2+}. These results are not surprising since presumably the tip-focused gradient is sufficient to saturate appropriate binding molecules such as calmodulin. However, what are the interacting proteins to which calmodulin binds? One example would be the actin binding proteins that are modulated by Ca^{2+} and calmodulin, including myosin and villin. Taken together it can be appreciated why there are not organized bundles of actin in the extreme apex and why streaming is markedly suppressed. A second example is ACA9 (Schiøtt et al. 2004), an autoinhibited, plasma membrane-localized Ca^{2+} pump, which is thought to be regulated by calmodulin, an observation consistent with the well-known role of calmodulin in the regulation of ion pumps in other systems (Snedden and Fromm 2001) (Fig. 4). We suspect that there are many other response elements that respond to Ca^{2+}/calmodulin, and that their identity will emerge from future work.

Recent work also supports the idea that calmodulin may interact with cyclic AMP in the regulation of pollen tube growth (Moutinho et al. 2001; Rato et al. 2004). Agents that either substitute for cAMP (8-Br-cAMP) or ac-

tivate adenylyl cyclase (forskolin) cause an increase in activated calmodulin, while inhibition of adenylyl cyclase (dideoxyadenosine) induces a decline in activated calmodulin. Rato et al. (2004) suggest that these interacting pathways participate in the regulation of apical secretion.

4.3.2 CDPKs

CDPKs comprise a large family of proteins in plants, and are prime candidates as possible Ca^{2+} response factors (see the chapter by Malhó, this volume). Curiously, thus far there has been relatively little work on presumptive pollen-specific forms of this protein. Estruch et al. (1994) provided the first evidence for a pollen-associated CDPK isoform that is necessary for pollen germination and tube growth. These authors favored the idea that this CDPK specifically participated in the regulation of the actin cytoskeleton. Subsequently, a CDPK from *Nicotiana alata* was identified that participates in the phosphorylation of stylar RNAses, and provisionally is involved in the incompatibility response in this species (Kunz et al. 1996). Moutinho et al. (1998a) used a fluorescent probe to spatially localize CDPK to the apical domain of the pollen tube. The pattern was similar to that of the tip-focused Ca^{2+} gradient, and was able to change its position in response to stimuli that cause reorientation of the pollen tube. A target for this presumptive CDPK is not known, but it is attractive to imagine that it participates in secretion (Moutinho et al. 1998a). Given the large size of the CDPK family, it seems likely that other pollen-specific members exist. This topic therefore deserves attention in future studies.

4.4 Small G-Proteins

Considerable excitement surrounds the idea that small G-proteins, namely Rops (Rho-related proteins of plants), regulate pollen tube growth, with an important aspect of that control mechanism involving the modulation of Ca^{2+} influx. Rops localize to the apex of the pollen tube where they appear to associate with the plasma membrane (Kost et al. 1999; Li et al. 1999; chapter by Hwang and Yang, this volume). Overexpression of these proteins causes the apex of the pollen tube to swell into balloon-shaped structures (Kost et al. 1999), whereas dominant negative forms of the protein or the injection of function-inhibiting antibodies to Rop block pollen tube growth (Li et al. 1999). Because these antibodies were shown to also eliminate the tip-focused Ca^{2+} gradient, the conclusion has been made that Rop regulates Ca^{2+} influx (Li et al. 1999). Despite this provocative conclusion, we hasten to note that the connection between Rop and Ca^{2+} may not be as compelling or direct as suggested. Several experimental conditions that block pollen tube elongation,

such as injection of BAPTA buffers, mild thermal shock, culture in caffeine, or treatment with elevated osmoticum, also eliminate the tip-focused Ca^{2+} gradient (Pierson et al. 1994, 1996). The fact that Rop inactivation blocks growth is indeed interesting, but the data thus far do not establish that it does so by first blocking Ca^{2+} influx. The results from the growth/Ca^{2+} relationship in oscillating pollen tubes indicate that growth defines the subsequent Ca^{2+} influx, and not the reverse (Messerli et al. 2000).

Despite these reservations, recent work establishes a firmer connection between Rop and Ca^{2+}, and also with the control of the actin cytoskeleton (Gu et al. 2005). In *Arabidopsis* pollen tubes, Rop1 seems to control tube growth through the coordinate activity of two interacting CRIB (Cdc42/Rac-interactive binding) proteins, called RIC3 and RIC4 (Gu et al. 2005). Experimentation suggests that RIC3 promotes Ca^{2+} influx, which may affect pollen tube growth through the modulation of F-actin dynamics. Support for these conclusions stems from the observation that cells expressing RIC3 germinate and express a tip-focused Ca^{2+} gradient at a lower extracellular [Ca^{2+}] than the untransformed controls. In addition, the results show that overexpression of RIC3 causes an apparent degradation of the fine actin filaments in the apical domain.

4.5 Turgor Regulation

Studies showing that the application of an increased level of osmoticum in the medium inhibits pollen tube growth suggest that osmotic regulation and the generation of turgor pressure are essential for cell elongation (Pierson et al. 1996). Messerli and Robinson (2003) have shown that an abrupt increase in turgor pressure, brought about by decreasing the osmolarity of the medium, can generate a brief increase in the growth rate. However, it does not follow that changes in turgor pressure underlie changes in growth rate, since studies using a pressure probe failed to detect a correlation between turgor pressure and growth rate, even during oscillatory growth (Benkert et al. 1997). Given the importance of maintaining turgor pressure, it is attractive to imagine that both K^+ and Cl^- play a key role (Fig. 4), since substantial fluxes of both ions have been measured. As noted by Zonia et al. (2002), the large efflux of Cl^- observed in pollen tubes could contribute to salt extrusion and turgor regulation, as has been shown in other systems, notably guard cells (Cosgrove and Hedrich 1991).

4.6 Cell Wall

Ions, especially Ca^{2+} and H^+, play important roles in the cell wall. The primary focus has been on Ca^{2+}, where its ability to cross-link carboxyl

residues on de-esterified pectins becomes an important factor in determining the structure and yielding properties of the cell wall (Fig. 4; chapter by Geitmann and Steer, this volume). Based on modeling in which the rate of growth was taken into consideration together with published data on the Ca^{2+} content of immature and mature cells walls, Holdaway-Clarke and Hepler (2003) calculated that the flux would amount to 35 pmol/cm^2/s. By contrast, the amount of influx needed to satisfy the intracellular tip-focused gradient was calculated to be only 1.7 pmol/cm^2/s. Based on this large difference and the observation that the measured flux could be as high as 20 pmol/cm^2/s, Holdaway-Clarke and Hepler (2003) reasoned that the cell wall requirement for Ca^{2+} dominated the influx detected by the ion-selective vibrating electrode.

H^+ can also have a profound effect on the cell wall structure and yielding properties. Generally in plants, while increased Ca^{2+} reduces growth, increased H^+ facilitate the process (Cassab and Varner 1988). During pollen tube growth, the action of pectin methyl esterase (PME), which has been secreted into the cell wall, causes the de-esterification of the methoxy residues on pectin (Bosch et al. 2005). This process also releases H^+, which can then downregulate PME because of the enzyme's sensitivity to lowered pH. It has been proposed that the balance between H^+ production and PME activity constitutes a key factor in controlling the oscillation of cell wall yielding properties and thus the oscillatory cell growth (Holdaway-Clarke et al. 1997). But other factors are also regulated by pH. For example, certain acidic PME isoforms are stimulated by lowered pH (Li et al. 2002), as are pectin hydrolases. In addition, there are wall-bound exo-α-glucanases, which have been identified in lily pollen tubes, that are regulated by pH (Kotake et al. 2000; chapter by Geitmann and Steer, this volume).

5 Perspectives

Ions occupy a central position in the control of pollen tube growth. Ca^{2+} attracts most attention, especially given that the tip-focused gradient resides precisely at the place where maximal growth is known to occur. It seems evident that this ion contributes to the localized secretion of cell wall components and cytoskeletal dynamics. We must also consider the likely possibility that future work will uncover a role for this ion in many other processes, especially as other factors such as pollen-specific CDPKs, are identified and characterized. H^+ also emerge as prime growth-controlling ions. Gradients in pH, established through the activity of H^+-ATPases and other H^+-pumping enzymes, serve as the basic energy source for transmembrane transport. But H^+ can also affect the actin cytoskeleton, and in addition modulate the structure and yielding properties of the cell wall. Finally, K^+ and Cl^- are

recognized for the central role they may play in turgor regulation and in determining the membrane potential.

The pollen tube continues to be one of the very best objects for examining ion gradients and fluxes. Its rapid growth probably accounts for the fact that the underlying ionic expressions are amplified and as a consequence easier to recognize than in slower growing cells. Continued work should markedly enlarge our understanding of ion regulation, and provide ideas that may apply broadly to other growing plant cells.

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Genomic and Molecular Analyses of Transporters in the Male Gametophyte

Heven Sze[1] (✉) · Sabine Frietsch[2] · Xiyan Li[1] · Kevin W. Bock[1] · Jeffrey F. Harper[2]

[1]Department Cell Biology & Molecular Genetics, HJ Patterson Hall, University of Maryland, College Park, MD 20742, USA
hsze@umd.edu

[2]Department of Biochemistry, Fleischmann Agriculture Bldg. #319, University of Nevada, Reno, NV 89557, USA

Abstract The major events of male reproductive development and function have been known for years, but the molecular and cellular bases of these processes are still poorly understood. Recent advances in cell biology coupled with molecular genetics and functional genomics are poised to offer tremendous opportunities to understand how membrane transport is integrated with male gametophyte development and physiology. Here we first propose the type of transporters necessary to affect the dynamics of Ca^{2+}, K^+, pH and others ions observed in polarized tip growth, and then show how pollen transcriptomics and molecular genetic tools are beginning to reveal the roles of specific transporters in microgametogenesis, pollen tube growth and male fertility.

1 Introduction

Little is known about the nutrients required for microspore proliferation and pollen maturation; however, it is well-established that pollen germination and tube growth depend on the continuous supply of ions and nutrients, like sucrose, Ca^{2+}, Mg^{2+}, K^+, NO_3^{2-}, SO_4^{2-} and boric acid (Brewbaker & Kwack 1963). Since the 1970s, ion currents and fluxes were recognized as being critical in establishing and/or maintaining polarity of pollen tube growth (Weisenseel et al. 1975; Jaffe et al. 1975). Studies using ratiometric ion imaging, bioluminescent indicators and vibrating probes have revealed the dynamic nature of Ca^{2+}, H^+ and K^+ during pollen tube growth (Holdaway-Clarke and Hepler 2003; Hepler et al., this volume). Most of these studies were conducted using pollen from lily, tobacco, or petunia plants. The results, summarized in Fig. 1A, suggest that ion gradients, oscillations and fluxes are mediated by pumps, channels and carriers that are regulated in a spatial- and temporal-manner in the growing pollen tube. However the identities and properties of the transporters involved are largely unknown. A first step to understanding the nutrition, physiology and development of the male game-

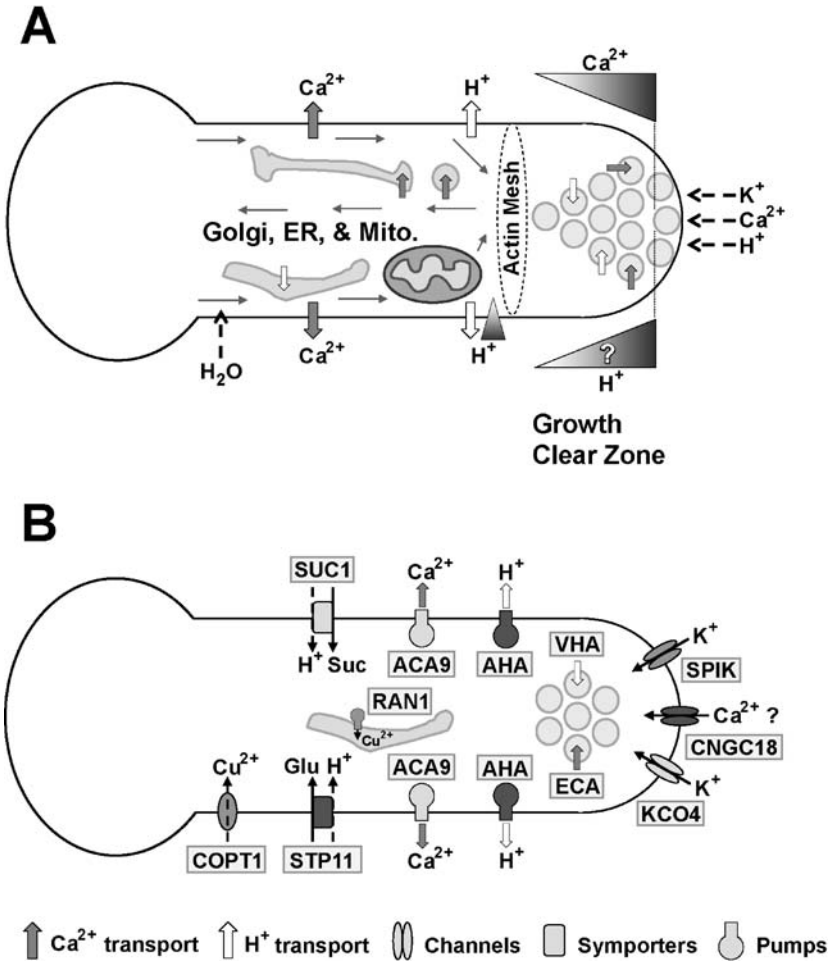

Fig. 1 Ion fluxes and specific transporters of pollen tubes. **A** Major ion fluxes and gradients detected in growing pollen tubes. A $[Ca^{2+}]_c$ gradient, high at the extreme tip, and a postulated pH_c gradient, acidic at the tip, of the pollen tube are denoted by *adjacent triangles*, respectively. Extracellular Ca^{2+} enters at the tip. Ca^{2+} is postulated to accumulate in internal stores and/or to be extruded across the plasma membrane. Release of internally stored Ca^{2+} could also increase $[Ca^{2+}]_c$. H^+ enters at the tip, and is pumped out at the shank. Extracellular K^+ enters at the tip. *Solid* and *dotted arrows* indicate active and passive transport, respectively. Active H^+ and Ca^{2+} transport is represented by *white-* and *grey-filled arrows*, respectively. *Line arrows* indicate direction of cytoplasmic streaming in the tube. Adapted from Holdaway-Clarke and Hepler (2003). **B** Selected pumps, cotransporters and channels identified in pollen tubes. Molecular studies have identified K^+ channels (SPIK and KCO4); putative Ca-permeable channel (CNGC18); Ca^{2+} pump (ACA9), and sugar symporters (SUC1 and STP11) at the plasma membrane. H^+ pumps AHA and VHA are presumed to be localized at the PM and endomembranes, respectively based on studies using sporophytic tissues. ECA Ca^{2+} pump and RAN1 Cu-ATPase are assumed to be on endomembranes. Cu transporter (COPT1) is likely localized to the PM

tophyte is to identify transporters expressed in pollen and to determine their specific functions.

2
Ion Current, Gradient and Oscillation During Pollen Germination and Tube Growth

2.1
Ca^{2+}

Ca^{2+} is the most prominent ion required for pollen germination and tube growth (Brewbaker & Kwack 1963). Depending on the plant species, extracellular Ca^{2+}, $[Ca^{2+}]_{ext}$, needed to support optimal tube growth *in vitro* varies from 0.1–5 mM (Steer & Steer 1989). At the apex, there is a tip-focused gradient of cytosolic free Ca^{2+}, $[Ca^{2+}]_c$ reaching 3–10 μM at the extreme tip and dropping sharply to basal levels of 150–300 nM within 20 μm from the apex (Fig. 1A, Hepler et al., this volume). The $[Ca^{2+}]_c$ gradient at the apex oscillates in phase with growth (Holdaway-Clarke et al. 1997; Messerli and Robinson 1997) and the oscillation, though large in magnitude (0.75–3 μM), remains at the extreme tip and is not propagated basipetally. Using yellow cameleon Ca^{2+} indicator, similar Ca^{2+} oscillations were observed at the tip of *Arabidopsis* pollen tube (Iwano et al. 2004). Changes of $[Ca^{2+}]_c$ within the apical dome also play a role in reorientation of the pollen tube (Malhó et al. 1995; Malhó and Trewavas 1996).

An intriguing question is how does pollen generate and maintain a $[Ca^{2+}]_c$ gradient that oscillates only at the extreme tip. With the electrical potential, negative inside (– 130 mV), and the pH gradients across the PM of plant cells (acid outside), Ca^{2+} is predicted to enter the cytosol passively via channels from the outside or from internal stores. Given the tip-focused Ca^{2+} gradient, it is likely that external Ca^{2+} enters via pathways concentrated at the pollen tip. Many experiments support this idea: (i) Autoradiography showed $^{45}Ca^{2+}$ is taken up at the tip (Jaffe et al. 1975); (ii) Mn^{2+} quenched indo-1 flouresence at the tip (Malhó et al. 1995); and (iii) vibrating electrode indicated influx of extracellular Ca^{2+} at the tip (e.g., Kuehtreiber & Jaffe 1990). $[Ca^{2+}]_c$ is most likely lowered by active Ca^{2+} pumps or H^+-coupled Ca^{2+} antiporters located on intracellular membranes and/or at the PM (Sze et al. 2000). Cytosolic Ca^{2+} oscillations are proposed to occur when Ca^{2+} release into the cytosol and its removal from the cytosol are temporally- and spatially-controlled by transporters localized at intracellular compartments and/or at the plasma membrane (Hepler et al., this volume).

The nature of the Ca^{2+} permeable influx and efflux pathways that create the tip-focused $[Ca^{2+}]_c$ gradient are poorly understood, though putative Ca^{2+} channel activities were recently reported. Suggested candidates for Ca^{2+}

permeable channels include stretch-activated ion channels as seen in fungal hyphae (Garrill et al. 1993), voltage-activated channels (Malhó et al. 1995) as observed in root hair cells (Very & Davies 2000), and cyclic nucleotide gated channels (CNGC) (Moutinho et al. 2001). Recently, Ca^{2+} conductance activated by symmetric positive or negative pressure was detected in lily pollen. The stretch-activated channel in the grain is restricted to a region where the tip emerges. Channel activity of the tube protoplast is lower in density and conductance than at the tip (Dutta & Robinson 2004). Spider venom, a blocker of stretch-activated ion channels, inhibited germination, tube growth and Ca^{2+} conductance. A hyperpolarization-activated inward Ca^{2+}-permeable channel was reported on the PM of *Arabidopsis* pollen (Wang et al. 2004) and of lily pollen protoplast (Shang et al. 2005). In lilies, the conductance was suppressed by trivalent cations, verapamil, nifedipine or diltiazem, and by calmodulin. The identities of these channels have yet to be determined at the molecular level. One possible candidate is CNGC18, a cation permeable channel, localized at the growing tip of *Arabidopsis* pollen (Frietsch S, Schroeder J, Harper JF, unpublished) (Sect. 4).

Assuming that pollen tubes are like other growing plant cells, it is likely that Ca^{2+} transporters on intracellular compartments participate in forming the tip-focused $[Ca^{2+}]_c$ gradient and Ca^{2+} oscillations. Secretory vesicles are abundant at the tip, thus they provide an enormous membrane area in close proximity to cytosolic Ca^{2+}. $[Ca^{2+}]_c$ increases at the extreme tip when extracellular Ca^{2+} enters the tube through activated PM-localized channels. Ca^{2+}-pumping ATPases (ACAs & ECAs) and H^+/Ca^{2+} antiporters (CAXs) are localized to the vacuole, ER, and perhaps in the Golgi and secretory vesicle/tubule abundant in growing cells, indicating that intracellular compartments serve as a rich source of stored Ca^{2+} (Sze et al. 2000). Chlorotetracycline fluorescence at the tip of growing pollen tube supports the idea for an apical gradient of Ca^{2+} associated with membranes (Reiss and Herth 1978; Malhó et al. 2000) but it has not been tested if $[Ca^{2+}]_m$ show the same type of dynamics observed for $[Ca^{2+}]_c$. Ca^{2+} release from the endomembrane vesicles through channels, such as those activated by hyperpolarization (e.g., Slow Vacuolar ion channel), and by ligands (IP_3, cADPR), could contribute to additional increase in $[Ca^{2+}]_c$. This rise in $[Ca^{2+}]_c$ would activate Ca^{2+} pumps and Ca^{2+}/H^+ antiporters in intracellular compartments and on the PM, thus lowering $[Ca^{2+}]_c$.

Ca^{2+} oscillations in pollen is thought to include temporally- and spatially-regulated "Ca^{2+} signatures" (Rudd and Franklin-Tong 2000; Sanders et al. 2002) that are involved in the specificity of the downstream responses, including turgor and cytoskeleton changes, and cell wall assembly. Despite recent electrophysiological evidence for calcium channel activity and isolation of the first Ca^{2+} transporter required for pollen tube growth and fertilization (Sect. 4), the molecular mechanisms controlling Ca^{2+} dynamics in polarized pollen tip growth remain a challenge.

2.2 pH and Oscillations

The role of pH in signaling is poorly understood in plants. *In vitro* pollen tube growth is optimal at pH 4.5–6, which is similar to root growth (Holdaway-Clarke et al. 2003). Recent studies using the pH indicator dye, BCECF-dextran, revealed the presence of a local pH gradient inside the pollen tube; an acidic domain estimated at pH 6.8 at the extreme tip in growing tubes, and an alkaline region of pH 7.5 at the base of the clear zone (Feijó et al. 1999). However the role of a pH_c gradient in tip growth is controversial. Several other studies showed an average pH_c of 7.1 with no standing pH gradient required for growth (Fricker et al. 1997; Parton et al. 1997; Messerli and Robinson 1998). Like Ca^{2+}, the internal pH (pH_i) oscillates at the apex (Feijó et al. 1999) and an acidic wave was observed to move basipetally (Messerli and Robinson 1998). How does oscillatory changes in pH occur? In plants, PM-localized pumps extrude H^+ to the outside, and V-ATPase and PPase pump H^+ into intracellular compartments (Sze et al. 1999). Vacuolar H^+-ATPases very likely affect pH in the cytosol, the compartments, and the outside, as these pumps are localized on Golgi, prevacuolar vesicles, and possibly secretory vesicles and endosomes. Unlike the case of Ca^{2+}, pH changes depend on a collaboration of co-transporters and channels in addition to H^+ pumps and passive H^+ fluxes. For instance, H^+ pumping into intracellular vesicles by a V-ATPase alone generates an electrical potential, positive inside, and a small change in ΔpH. However, anion influx (e.g., Cl^-) presumably via a voltage-activated anion channel will dissipate the charge difference and increase H^+ pumping to form an acidic domain at the lumenal side (Sze 1985). Conversely, activation of an H^+/K^+ exchanger at the vacuolar membrane where H^+ moves back to the cytoplasmic face, could raise the pH at the lumen face or locally acidify the cytosolic side. The local gradients would form albeit transiently due to the mobility of the H^+ ion and cytosolic buffering.

Thus, a pH oscillation would be formed by the temporal activation and inactivation of transporters that are localized on intracellular membrane and on the PM. The transporters include proton pumps, ion channels, H^+-coupled cotransporters or proton leak pathways. As K^+ is the major osmotic ion in cells, K^+ channels and H^+/K^+ exchangers are postulated to be prominent players. PM and vacuolar H^+-ATPases are highly expressed in mature pollen and in pollen tubes (Padmanaban et al. 2004; Dettmer et al. 2005; Sect. 3), though the identity and spatial location of ion channels, H^+-coupled cotransporters or proton leak pathways are poorly defined.

2.3
K^+

As the most abundant ion in plant cells (where $[K^+]_c$ is maintained at ~ 75 mM), K^+ serves various functions, including osmoregulation, cell expansion, cell movement, enzyme/protein activation, stress tolerance and modulation of the electrical potential. Pollen tube growth can be sustained with 0.1 or 1 mM K^+_{ext} indicating that K^+ is taken up via an energy-dependent H^+/K^+ symporter, and via K^+_{in} channels (Mouline et al. 2002). Various K^+ channel activities have been detected in protoplasts from pollen using the whole cell patch-clamp or single channel recording. These include (i) a hyperpolarization-activated cation channel (HACC), like an inward K^+ conductance that is insensitive to $[Ca^{2+}]_c$ but stimulated by acidic pH_{ext} (Fan et al. 2001; Mouline et al. 2002); (ii) depolarization-activated cation channel (DACC) that conducts K^+ outward (Fan et al. 2003); (iii) voltage-insensitive cation channel (VICC) that conducts K^+_{in} (Becker et al. 2004), (iv) voltage-insensitive and stretch-activated K^+, and (v) a spontaneous K^+ conductance (Griessner and Obermeyer 2003; Dutta and Robinson 2004). Genetic and functional studies have identified the first K^+ channels in pollen, SPIK/AKT6 as a Shaker K^+ inward rectifier (Mouline et al. 2002), and KCO4 as a voltage-insensitive K^+ channel (Becker et al. 2004) (Sect. 4). Nearly nothing is known about K^+ transporters on the vacuolar membrane of the vegetative cell, though vacuoles are very dynamic in growing pollen tubes (Hicks et al. 2004).

2.4
Other Ions-Chloride & Borate

Cl^- is considered a micronutrient that is needed for plant growth at an external concentration of $\sim 50\ \mu$M. Its major role is thought to regulate electrical potentials, and so influence turgor and cell volume (Ward et al. 1995). Interestingly, Cl^- is not required for *in vitro* pollen tube growth, suggesting its major role is in electrical signaling. Inorganic anions enter cells against an electrochemical gradient mediated by PM-localized H^+-anion symporters. When signals, like high $[Ca^{2+}]_c$, stimulate opening of an anion channel, anion efflux causes membrane depolarization which can lead to downstream physiological responses (e.g., Ward et al. 1995; Cho and Spalding 1996).

The relative contribution of anion flux, particularly Cl^- in pollen tube growth is currently controversial. Anion channel blockers, like DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), NPPB (5-nitro-2-(3-phenylpropylamide)benzoic acid) or niflumic acid, inhibited pollen germination and apparent Cl^- efflux (Zonia et al. 2002; Matveyeva et al. 2003). Cl^- efflux that oscillated in phase with growth was reported at the extreme apex (Zonia et al. 2002) but the selectivity of the microelectrode for Cl^- to other anions

(< 10 fold) or to MES (2.4 fold) was low, raising the possibility that H^+ influx contributed in large part to the signal (Messerli et al. 2004; Hepler et al., this volume). Inhibitor studies would suggest that Cl^- or other anion transport are involved in pollen germination and tube growth. Cl^- channel activity was, however, not detected by whole cell patch clamping under any condition tested (Dutta & Robinson 2004). Clearly, further studies are needed to determine the location of anion flux and the specific protein(s) mediating the transport.

Although external boric acid has long been recognized as an essential element for *in vitro* pollen tube growth, a biochemical role of boron in plant nutrition was recently established (Blevins and Lukaszewski 1998). One role is to stabilize cell wall structures. L-Fucose-deficient *Arabidopsis* mutants show only 50% cross-linked pectin and have a dwarfed phenotype that is reversed by exogenous borate and L-fucose (O'Neill et al. 2001). These results are consistent with the idea that borate ester connects and dimerizes rhamnogalacturonan II, a subclass of pectin. The finding that *Picea* pollen germinated in boron-deficient medium accumulated acidic pectin at the pollen tip (Wang et al. 2003) would support this idea. Combined with the requirement for extracellular fluxes of Ca^{2+} and pH in pollen tip growth, it has been proposed that binding of Ca^{2+} and borate to pectin may alter the mechanical properties of the apical cell wall (Holdaway-Clarke et al. 2003). Uncharged boric acid may enter the cell by diffusion, and borate is then supplied to walls via a PM-localized BOR1 efflux transporter (Takano et al. 2002).

3
Integrating Transport with Male Gametophyte Development and Function

One powerful approach to identify transporters that function in pollen development is through transcriptomics (Twell et al., this volume). The pollen transcriptome revealed for the first time most of the genes expressed in the mature pollen. Moreover, a unique dataset of four stages of male gametophyte development enables developmental analyses of gene expression (Honys and Twell 2004). Identification of specific transporter genes expressed in developing pollen is providing insights for strategic mutant analyses to integrate the roles of transporters in pollen development, pollen maturation, or post-pollinations events.

3.1
Identifying Transporter Genes Specific to Pollen in Arabidopsis

To identify transporter genes expressed in pollen, Bock et al. (2006) compiled a list of all known and predicted transporters from the *Arabidopsis thaliana* genome. Transport proteins are highly conserved from bacteria to eukaryotes;

Table 1 Partial list of transporter genes that are specifically (S) or preferentially (P) expressed in male gametophyte. The pollen transcriptome from 4 stages of development (Honys and Twell 2004) was compared with transcriptome data of 12 sporophytic tissues. MaxPo indicates the maximum expression level observed in any stage of male gametophyte; MaxSp refers to the maximum expression signal in any sporophytic tissue. Preferential expression was defined as genes showing a Fold Change or ratio pf MaxPo/MaxSp of > 3 (Bock et al. 2006)

AGI Name	TC #	FAMILY	Protein Description	PROTEIN	Pol	Max Po	Max Sp	Fold C	Clus
At2g25600	1.A.1	VIC	potassium channel	AtSPIK	S *	1852	0	Spec	1
At1g19780	1.A.1	VIC	put. cyclic nucleotide and calmodulin-reg. ion channel	AtCNGC8	S *	1153	0	Spec	2
At5g14870	1.A.1	VIC	put. cyclic nucl and calmodulin-reg. ion channel	AtCNGC18	P *	1099	95	11.5	2
At1g15990	1.A.1	VIC	put. cyclic nucl. and calmodulin-reg. ion channel	AtCNGC7	S *	426	0	Spec	2
At3g48010	1.A.1	VIC	put. cyclic nucl. and calmodulin-reg. ion channel	AtCNGC16	S *	420	0	Spec	2
At4g01470	1.A.8	MIP	putative tonoplast intrinsic protein 3 gamma	AtTIP1.3	P *	4787	27	176.7	18
At5g37810	1.A.8	MIP	NOD26-like intrinsic protein	AtNIP4.1	P *	1796	18	97.5	5
At3g47440	1.A.8	MIP	putative tonoplast intrinsic protein	AtTIP5.1	S *	1616	0	Spec	3
At5g23270	2.A.1.1	MFS	monosacc.-H^+ symporter	AtSTP11	P *	3814	140	27.3	1
At1g07340	2.A.1.1	MFS	monosacc.-H^+ symporter	AtSTP2	P	3403	60	57.1	29
At3g05150	2.A.1.1	MFS	glucose transport family, AtERD6 homol.	At3g05150	P *	2510	320	7.9	1

* indicates genes assigned by Pina et al. (2005) as pollen-specific or pollen-enriched in mature pollen. Cluster number (Clus) refers to distinct expression pattern of each gene assigned by Honys & Twell (2004)

Table 1 (continued)

AGI Name	TC #	FAMILY	Protein Description	PROTEIN	Pol	Max Po	Max Sp	Fold C	Clus
At1g50310	2.A.1.1	MFS	monosacc.-H^+ symporter, glucose-spec	AtSTP9	P *	1923	165	11.7	22
At3g03090	2.A.1.1	MFS	xylose transporter homolog	At3g03090	P *	1880	287	6.6	1
At5g28470	2.A.17	POT	proton-dependent oligopeptide transport	At5g28470	S *	1648	0	Spec	25
At4g35180	2.A.18	AAAP	putative lys/his transporter	AtLHT7	P *	5640	477	11.8	1
At1g71680	2.A.18	AAAP	Put. lys/his transporter	AtLHT8	S	4104	0	Spec	20
At5g25430	2.A.31	AE	putative boron transporter	At5g25430	P *	1497	46	32.2	1
At3g17630	2.A.37	CPA2	Put. cation-H^+ exchanger	AtCHX19	P	3133	107	29.2	22
At2g28180	2.A.37	CPA2	Put. cation-H^+ exchanger	AtCHX8	S *	3086	0	Spec	1
At2g13620	2.A.37	CPA2	Put. cation-H^+ exchanger	AtCHX15	S *	1585	0	Spec	2
At1g79400	2.A.37	CPA2	Put. cation-H^+ exchanger	AtCHX2	P *	1198	69	17.2	2
At5g01690	2.A.37	CPA2	Put. cation-H^+ exchanger	AtCHX27	P *	1084	268	4.1	2
At4g18790	2.A.55	Nramp	Put. ion metal transporter	AtNRAMP5	P *	1477	35	42.3	2
At2g07560	3.A.3	P-ATP	put. PM P3A-H^+-ATPase	AtAHA6	S *	8417	0	Spec	18
At1g80660	3.A.3	P-ATP	put. PM P3A-H^+-ATPase	AtAHA9	S *	6379	0	Spec	18
At3g42640	3.A.3	P-ATP	put. PM P3A-H^+-ATPase	AtAHA8	P *	6225	162	38.4	2
At3g21180	3.A.3	P-ATP	putative Ca^{2+} P2B-ATPase	AtACA9	P *	2781	349	8.0	3
At2g22950	3.A.3	P-ATP	putative Ca^{2+} P2B-ATPase	AtACA7	S *	2530	0	Spec	18

* indicates genes assigned by Pina et al. (2005) as pollen-specific or pollen-enriched in mature pollen. Cluster number (Clus) refers to distinct expression pattern of each gene assigned by Honys & Twell (2004)

Table 1 (continued)

AGI Name	TC #	FAMILY	Protein Description	PROTEIN	Pol	Max Po	Max Sp	Fold C	Clus
At1g54280	3.A.3	P-ATP	put. aminophospholipid translocase, P4-type	AtALA6	P *	1449	170	8.5	3
At3g13900	3.A.3	P-ATP	put. aminophospholipid translocase, P4-type	AtALA7	P *	1110	90	12.3	3
At1g26130	3.A.3	P-ATP	put. aminophospholipid translocase, P4-type	AtALA12	P *	861	154	5.6	3
At5g59040	9.A.12	Ctr2	copper transporter	AtCOPT3	S	3973	0	Spec	29

* indicates genes assigned by Pina et al. (2005) as pollen-specific or pollen-enriched in mature pollen. Cluster number (Clus) refers to distinct expression pattern of each gene assigned by Honys & Twell (2004)

thus, they can be classified into functional families according to the Transport Classification (Busch and Saier 2004). Using databases of genes encoding plant membrane proteins, including the Arabidopsis Membrane Protein Library (AMPL) (Ward 2001); PlantsT (Tschieu et al. 2003) and ARAMEMNON (Schwacke et al. 2003), a comprehensive list of all known or predicted transporters was assembled. Of 1106 classified transporter genes present on the ATH1 chip, ~ 746 are expressed in pollen (Bock et al., accepted; Twell et al., this volume). By comparing the normalized gene expression level of pollen at four developmental stages (Honys & Twell 2004) to that of 12 sporophytic tissues, more than 120 genes that are specifically or preferentially expressed throughout pollen development were identified (Table 1). Genes that were present in pollen but absent in any sporophytic tissue were labeled as "pollen-specific". Genes that were expressed at least 3-fold higher level in any stage of pollen relative to the maximum expression in any sporophytic tissues were marked as "preferentially-expressed". In a study of mature pollen transcriptome, Pina et al. (2005) assigned 94 transporter genes as pollen-specific or pollen-enriched.

In spite of different methods used to analyze two pollen transcriptomes, 51 genes were identified by both groups as either specifically or preferentially expressed in mature pollen (see Table 1 for partial list). Highly expressed genes include PM H^+ pumps (AHA6, AHA8, AHA9), and Ca^{2+} pumps, (ACA9, and ACA7). Prominent channels include the SPIK K^+ channel, several CNGC channels and putative aquaporin TIP5.1. Putative cation/H^+ exchangers (CHX8, CHX15, CHX19), and one encoding a putative boron transporter (At5g25430) are preferentially expressed. Among metabolite transporters, several members of sugar transporters in the MFS superfamily (STP11), oligopeptide (At5g28470) and amino acid (LHT7, LHT8) transporters stand out. Most of these are expressed at the tricellular stage or in mature pollen grain. Analyses by Bock et al. (in press) showed that 5.2% and 7.1% of all genes expressed in microspores and mature pollen, respectively, are classified transporter genes. Furthermore, the proportion of transporter genes that are pollen – preferential (or specific) increased from 13.7% in microspore to 20.9% or 107 genes in tricellular and mature pollen (Honys and Twell 2004; Bock et al., in press; Twell et al., this volume). Thus the proportion of pollen preferential transporter genes being expressed increases as pollen matures, underscoring the significance of transport in the maturation and functions of the male gametophyte.

3.2
Early and Late Pollen Expressed Genes

While the transcriptome analyses indicates that pollen show expression for all the different classes of transporter genes that are also expressed in sporophytic tissues, analyses of individual gene families provides insights into

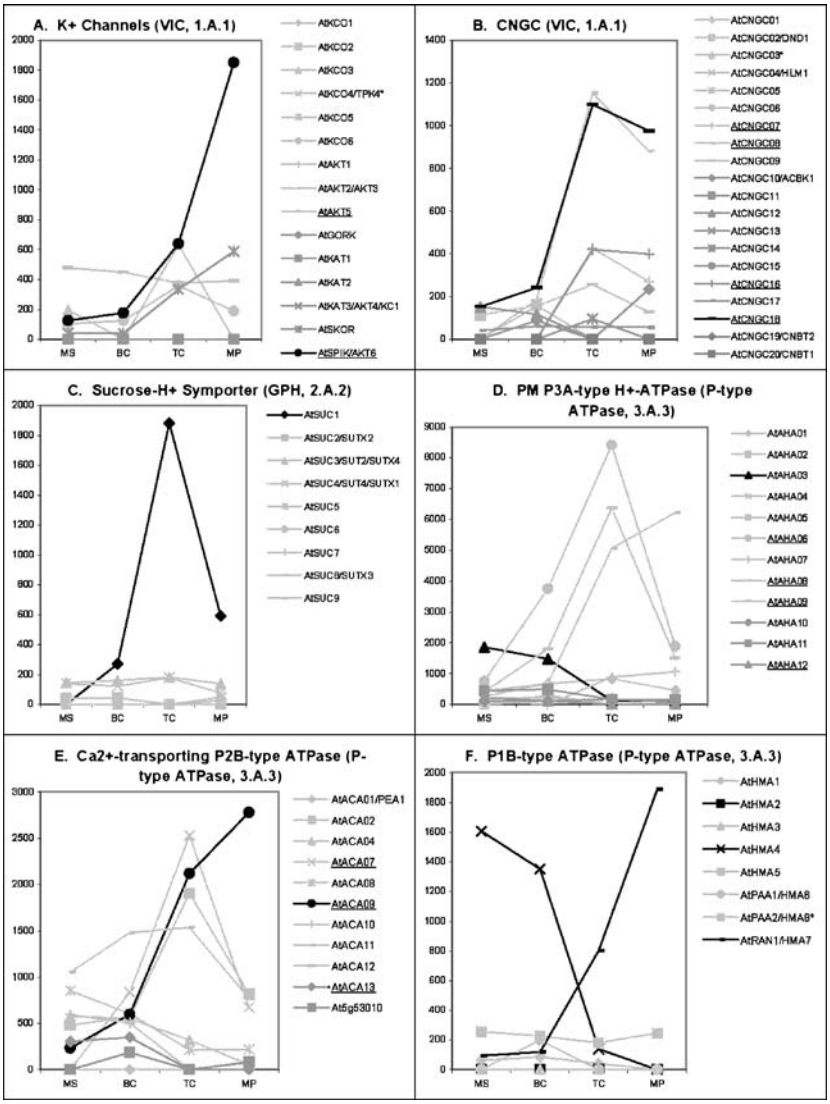

Fig. 2 Discrete subset of genes within selected gene families are expressed in male gametophyte in a developmentally-regulated manner. Relative expression of all genes within a gene family was monitored at the microspore (MS), bicellular (BC), tricellular (TC) and mature pollen (MP) stages. Each gene is identified by the given name when available or by the AGI number. *Underlined* gene name indicates those that are specifically or preferentially expressed in pollen. *Black line* highlights genes that have been genetically or functionally characterized. **A** VIC K^+ channel; **B** VIC Cyclic Nucleotide-Gated ion Channel (CNGC); **C** GPH Sucrose-proton symporter/sucrose transporter (SUC), TC# 2.A.2; **D** Plasma membrane P3A-type H^+-ATPase, TC# 3.A.3; **E** Calmodulin-regulated Ca^{2+}-transporting P2B-type ATPase, TC# 3.A.3; **F** Cu^{2+}-transporting and $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$-transporting P1B-type ATPase, TC# 3.A.3

potentially significant isoform-specific patterns of developmental regulation. For example, the PM H^+ pump AHA3 (non-pollen specific) gene is the only AHA member expressed early in development at the microspore and bicellular stage. In contrast, AHA6, AHA8 and AHA9 are expressed late in development at the tricellular pollen stage at 5–7-fold higher levels than other AHAs (Fig. 2). Among autoinhibited Ca^{2+} pumps, several ACAs are expressed early in development, whereas ACA7, ACA2 and ACA9 are activated as pollen matures.

Expression patterns evaluated by other independent methods provide strong support for the normalized transcriptome results: (i) A portion of the pollen-specific or preferential genes has been verified by PCR amplification of reverse-transcribed pollen messages (Sze et al. 2004); (ii) The early and late pollen-expressed genes suggested by the microarray results have been confirmed by promoter::Gus staining of the anther or pollen at different floral stages for several CHX genes (Bock et al., in press); and (iii) differential expression of discrete gene members seen in transcriptomics were observed before by in situ hybridization or immunohistochemical staining. For instance, STP2 mRNA and protein were localized in the microspores but not found in tricellular or mature pollen (Truernit et al. 1999). In contrast, STP11 protein is exclusively in the pollen tube but not in the grains (Schneidereit et al. 2005). Transcriptomic analyses showed that STP11 message peaks at the tricellular stage; these results support the idea that many messages are stored until pollen germination.

The distinction of early and late pollen expressed genes is striking, indicating a sharp repression of certain genes at the bicellular stage, and an activation of other late transporter genes at the same time. It is then likely that early pollen-expressed genes are involved in microspore expansion and proliferation, whereas late-pollen expressed genes participate in pollen maturation, and in post-pollination events (Schiott et al. 2004; Twell et al., this volume).

4
Genetic and Functional Analyses of Transporters in Pollen Development and Tube Growth

The expectation is that most of the 746 pollen-expressed transporters in *Arabidopsis* will eventually be found to contribute to the development or fitness of pollen. The following are examples of gene knockout approaches that combined with other results have begun to reveal insights into pollen transporter functions. Although gene knockouts provide powerful tools, additional insights are expected from experimental strategies that include silencing, overexpression, and the use of dominant negative transgenes (Twell et al., this volume; Guermonprez et al., this volume).

4.1 Sugar Transporters

A large number of sugar transporters are specifically or preferentially expressed in the male gametophyte underscoring the demand for energy and carbon nutrient during microgametogenesis. STPs are monosaccharide/H^+ symporters at the plasma membrane and differential expression of STP2 and STP11 during microgametogenesis (Sect. 3), suggest that distinct STPs are used to take up monosaccharides at different stages of development (Truernit et al. 1999; Schneidereit et al. 2005). Other pollen-specific STPs (e.g., STP6, STP9) are co-expressed late in pollen development (Schneidereit et al. 2003; Scholz-Starke et al. 2003). This redundancy could account for the lack of phenotype in knockout mutants, such as in *stp6*.

Only one sugar transporter mutation has so far been shown to result in a pollen phenotype. A T-DNA gene disruption of SUC1 (*hap3*) was found to be completely male sterile (Johnson et al. 2004). *In vivo* cytological analysis indicated that mutant pollen tubes failed to enter the transmitting tract. SUC1 belongs to a family of sucrose/H^+ symporters localized at the PM (Stadler et al. 1999). Transcriptome data indicates it is the only SUC gene that is highly expressed relative to six other SUC family members in tricellular pollen (Fig. 2C). It is interesting that in spite of several monosaccharide/H^+ symporters in developing pollen, the genetic studies would suggest that a sufficient quantity of sucrose is critical to support pollen tube growth.

4.2 Proton Pumps

The expected function of multiple plasma membrane proton pumps (AHA, autoinhibited H^+-ATPase) in *Arabidopsis* is to energize the plasma membrane with 1) a proton gradient that drives nutrient co-transport systems, and 2) an electrical potential that drives ions through channels for both nutritional and signaling functions. The first null mutation reported for a plant AHA (*aha3*) was found to cause male sterility (Robertson et al. 2004). A cytological analysis indicated that the defect in *aha3* occurred early in pollen development but whether the defect in *aha3* is related to defects in signaling and/or nutrient uptake has not been determined.

The *aha3* loss-of-function phenotype provides an important new tool for investigating the structure and function of plant P-type proton pumps. Using *aha3* (–/+) plants, complementation was observed by the expression of a wild-type AHA3, but not a mutant AHA3 harboring a mutation of the penultimate residue in the C-terminal end (T948A) (Robertson et al. 2004). This mutation destroys a phosphorylation site critical to 14-3-3 binding. Since the binding of 14-3-3 can hyper-activate a plant P-type proton pump *in vitro*, it has been proposed as a key regulatory feature for controlling proton pump

activity *in planta* (Palmgren 2001). Strong evidence supporting this hypothesis is now provided by the failure to obtain complementation with the T948A mutant pump. These complementation experiments provide evidence that 14-3-3 is essential to pollen development, with at least one target being the plasma membrane proton pump.

The pollen sterile *aha3* null phenotype indicates that AHA3 provides an essential function that is not redundant with any of the other 11 AHA isoforms, or other proton pumping enzymes in plants (e.g., V-type proton pumps). Pollen transcriptome shows that *AHA3* appears to be the most highly expressed of the AHA genes in early pollen development (Fig. 2). At later stages of pollen development, different isoforms (AHA6, AHA8 and AHA9) are expressed at much higher levels. Whether one or more of these isoforms are involved in regulating the pH_c and pH_{ext} of growing pollen tubes has yet to be established.

Another proton pump, the vacuolar-type H^+-ATPase (VHA) acidifies various intracellular compartments, including the vacuole, ER, Golgi and undefined vesicles. The growing pollen tube is an exciting model to study the roles of the V-ATPase given its proposed functions in protein sorting, vesicle trafficking and membrane fusion (Sze et al. 1999, 2002; Padmanaban et al. 2004; Dettmer et al. 2005). In sporophytic tissues, the proton motive force energizes co-transport of ions and metabolites, and so is important for osmoregulation, cell expansion, and stress tolerance. However, other studies suggest that the functions of V-ATPase is much more diverse (Sze et al. 2002). In *Arabidopsis*, V-type ATPase complex of 12 subunits are encoded by 26 genes, most of which are highly expressed in pollen. Disruption of *VHA-A* (*vha-A*), a single copy gene for subunit A causes complete male and partial female gametophytic lethality (Dettmer et al. 2005). Evidence from RT-PCR analysis suggests small levels of *VHA-A* transcript in *vha-A* mutants. Nevertheless cytological analysis revealed abortion of *vha-A* pollen during development and Golgi stacks with abnormal morphologies after the first mitosis in mutant pollen grains, suggesting a crucial role of *VHA-A* in Golgi organization and protein trafficking (Dettmer et al. 2005).

The pollen lethal phenotype of *vha-A* suggests that proton pump activity of V-ATPase cannot be compensated by other H^+ pumps, such as the PPase AVP2 that is expressed during pollen development and that also targets to the Golgi (Dettmer et al. 2005).

4.3 K^+ Channels (VIC Family)

Of 15 genes encoding K^+ channels in the family of Voltage Gated-Ion Channels (VIC), only a few show relatively high expression in pollen according to transcriptome data (Fig. 2). SPIK, a Shaker channel, is highly expressed in male gametophyte late in development. Mutant pollen with gene disruption

in SPIK/AKT6 (At2g25600) showed decreased rate of tube growth in $[K^+]_{ext}$ ranging from 5 μM to 1 mM, and reduced hyperpolarization activated K^+_{in} current (Mouline et al. 2002). The channel properties suggest SPIK is active at the PM and that it mediates bulk transport involved in K^+ nutrition. The *spik-1* mutation did show a general decrease in pollen fitness, suggesting that SPIK mediates K^+ uptake in growing pollen tube, and has a role in pollen tube development and pollen competitive ability. The residual K^+-dependent germination in the *spik-1* mutant is likely due to other K^+ permeable channels. However, gene disruption of another pollen-specific K^+_{in} channel, TPK4 (KCO4), did not appear to alter pollen tube growth (Becker et al. 2004), though the ratio of the instantaneous current to the steady state current was reduced in the mutant pollen tube. TPK4 may be related to the spontaneously activating K^+ channel characterized in lily pollen (Dutta and Robinson 2004). Localized to the PM, TPK4/KCO4 is voltage-insensitive and is modulated by Ca^{2+} and pH, suggesting it has a role in K^+ homeostasis and voltage control (Becker et al. 2004).

The roles of four other K^+ channels of the VIC family expressed in pollen (Fig. 2) are not known though SKOR is most likely an outward K^+ rectifier as shown in xylem parenchyma cells (Very & Sentenac 2003). The roles of other putative K^+ transporters, including a K^+/H^+ symporter (KUP), K^+ $(Na^+)/H^+$ antiporters (CHX and NHX families), and other cation channels potentially permeable to K^+ (e.g., Glutamate receptors and CNGC) need to be investigated as a few members of these families are highly expressed in developing pollen (Table 1; Bock et al., in press).

4.4 Metal Transporters

Although the role of heavy metals in pollen development is not understood yet, their importance is highlighted by nutrition and genetic studies, and by the pollen transcriptome. For example, copper-deficient plants failed to produce grains possibly due to a defect in pollen formation (Graham 1997). It is thus interesting to find copper and other metal transporter genes (e.g., COPT3, COPT1) expressed at early and at late stages of pollen development (Bock et al., in press). Functional expression in yeast suggested COPT1 mediates Cu uptake at the PM, and this is confirmed by decreased Cu uptake and Cu content of transgenic *Arabidopsis* plants expressing anti-sense COPT1 (Sancenon et al. 2004). About 12% of the pollen grains from anti-sense plants showed slightly deformed morphology suggesting that Cu may be important for formation of the pollen wall.

The *Arabidopsis* genome encodes eight heavy metal-transporting P-type ATPases (HMA). The first loss-of-function mutant (*hma7/ran1*) was isolated in a genetic screen for ethylene response mutants (Woeste and Kieber 2000). Homozygous *hma7/ran1* plants show a copper deficiency and normally die

as young seedlings. If grown on agar media, mutants can survive till the reproductive phase but plants develop small flowers and are completely sterile. While this mutant clearly has a whole plant phenotype, there is also evidence for a pollen autonomous function of *hma7/ran1*. When pollen from plants heterozygous for *hma7/ran1* were crossed to wild type, there was a reduced transmission of the *hma7/ran1* mutant allele (i.e., segregation distortion). The *hma7/ran1* phenotype indicates that HMA7 provides essential function in plant development. Assuming RAN1 is localized to endomembrane compartments (e.g., Golgi or ER), the mutant phenotype suggests that Cu uptake into intracellular compartments is critical for yet undetermined aspects of pollen development or tube growth.

A double null mutation of isoforms HMA2 and HMA4, which are putative zinc translocating pumps, also result in severe growth defects, including anthers that do not produce any pollen (Hussain et al. 2004). These growth defects can be reversed by growing plants on high levels of zinc. HMA2 and HMA4 are expressed in vascular tissues, and HMA2 was localized to the PM. Both pumps are thought to have a role in regulating Zn^{2+} homeostasis in the whole plant.

HMA2 and HMA4 appear to have some level of functional redundancy or complementary activity since the sterility phenotype was only observed in the double knockout (Hussain et al. 2004). Expression profiling indicates that HMA4 and HMA7/RAN1 are the highest expressed isoforms at the early and late stages of developing pollen, respectively (Fig. 2F). Although expression for HMA2 was not detected in two independent microarray data sets, a positive result observed for a promoter-GUS reporter analysis suggests that HMA2 is expressed at an early stage of pollen development, but not in mature pollen (Hussain et al. 2004). It remains unclear if the male sterility of *hma2 hma4* double mutants is primarily caused by a general perturbation of Zn^{2+} homeostasis in the whole-plant or whether these pumps have an essential pollen-specific function as well.

4.5 Calcium Pumps

The *Arabidopsis* genome encodes 14 P-type calcium pumps (10 ACAs, autoinhibited Ca^{2+}-ATPase and 4 ECAs, ER-type Ca-ATPases) that are proposed to lower $[Ca^{2+}]_c$ by extruding Ca^{2+} into intracellular compartments or outside the cell (Sze et al. 2000). The first null mutation reported for a plant ACA (*aca9*) was found to cause partial male sterility (Schiott et al. 2004). The primary defect was a failure of *aca9* pollen to discharge sperm into the synergid. While mutant pollen tubes were able to reach ovules, they failed to complete fertilization in more than 50% of the interactions. Evidence indicates that the ACA9 pump is primarily located at the plasma membrane. A plasma membrane Ca^{2+} pump may either function in signal transduction by helping to

control the magnitude or duration of a Ca^{2+} signal (signaling function), or in cell wall biogenesis by pumping Ca^{2+} directly into the wall (nutritional function), or both. The fact that ACAs are activated by Ca^{2+}/calmodulin, indicates that they are directly connected to Ca^{2+} signaling.

The observation that the *aca9* null phenotype does not result in complete male sterile phenotype leaves open the question of whether other isoforms provide some low level of functional redundancy. In mature pollen, expression profiling studies show that *ACA9* is more highly expressed than any other ACA type calcium pump, including ACA2, ACA7 and ACA8 (Fig. 2). The expression of ACA8, a known plasma membrane Ca^{2+} pump (Bonza et al. 2000), under the control of the ACA9 promoter was shown to complement the *aca9* null mutation. While this indicates that *Arabidopsis* pollen do express pumps that have the potential to be functionally redundant, expression levels are not sufficient to completely compensate for the loss of the most abundant isoform, ACA9.

Interestingly, pollen-expressed ACA9 is also preferentially expressed in roots based on the transcriptome data of several sporophytic tissues (Bock K, Sze H, unpublished). As root hairs are known to elongate by tip growth, it is tempting to speculate that ACA9 performs a similar function in tip growth of both pollen tube and root hairs.

Pumps that load Ca^{2+} into endomembrane compartments also influence pollen tube growth. Although several ECA genes are expressed in pollen, homozygous mutants of ECA3 gene alone reduced pollen tube growth *in vitro* (Li X, Harper JF, Sze H, unpublished). Results suggest ECA3 affects lumenal $[Ca^{2+}]$ in compartments distinct from the ER where ECA1 and ACA2 are localized. It is possible that *eca3* mutants altered one or more of the following: (i) $[Ca^{2+}]_c$ dynamics needed for signaling; and (ii) lumenal $[Ca^{2+}]$ required to activate enzymes/proteins involved in protein sorting, modification or cell wall biosynthesis.

4.6 Cyclic Nucleotide-Gated Channels

CNGCs in plants, like animal homologs, are non-selective cation channels that are Ca^{2+} permeable (Talke et al. 2003). The first null mutation reported for a plant CNGC (*cngc2*) resulted in a dwarf plant with a lesion mimic/pathogen response phenotype, as well as poor fertility (Clough et al. 2000). While a specific role of CNGC2 in pollen has not been investigated, this gene is highly expressed at early stages of pollen development. In contrast to cngc2's weak fertility phenotype, a complete male sterile phenotype results from a null mutation of isoform CNGC18 (Frietsch S, Schroeder J, Harper JF, unpublished). Two independent T-DNA insertions in *CNGC18* were found to completely block pollen transmission of the mutant allele. A cytological analysis indicated that the primary defect was a failure of *cngc18* pollen

to undergo directional growth into the transmitting tract of the pistil. Evidence from a GFP tagging strategy suggests that CNGC18 has a tip-focused plasma membrane location. While the ion conductance properties of CNGC18 have not been directly tested, CNGCs are thought to be Ca^{2+} permeable, nonspecific cation channels (Leng 1999; Talke et al. 2003) that are activated by cyclic nucleotides and inhibited by the binding of Ca^{2+}/calmodulin (Talke et al. 2003; Hua et al. 2003). The apparent tip-focused location of CNGC18 suggests that this channel may function in regulating tip-localized Ca^{2+} signals that coordinate the directional growth machinery (Fig. 1B).

The *cngc18* null phenotype indicates that CNGC18 provides an essential function that is not compensated by any of the other 6 or 7 pollen-expressed CNGC isoforms (Fig. 2B). In mature pollen, *CNGC18* and CNGC8 are the two most highly expressed *CNGC*s, with other isoforms showing less than half of their expression levels. Since some CNGCs are thought to form heteromultimers, multiple isoforms expressed in plant pollen may form a highly diverse set of channels with different ion permeation or gating properties. Nevertheless, a function for these additional pollen isoforms has not yet been established.

5 Perspectives

Genetic studies are just beginning to provide valuable insights on the roles of specific pumps, carriers and channels in pollen nutrition, development and tube growth. Remarkably, almost 70% (746) of classified transporter genes are expressed in the male gametophyte of *Arabidopsis*. Most of these are expected to be expressed in the single vegetative cell that develops into the growing pollen tube, thus the developmentally-regulated expression of distinct transporters give clues for strategic functional analyses of each mutant. We anticipate that this system will provide a paradigm for transporter functions in general, and pollen development in specific. It is important to note that pollination and post-pollination events trigger pistil responses and guidance cues (Johnson et al., this volume) that conceivably modulate ion fluxes and gradients, and so orient pollen tube growth. Furthermore new methods are emerging to analyze *in vivo* and semi-in vivo pollen tube growth (Higashiyama and Inatsugi, this volume) and to image ion dynamics in targeted cells (Iwano et al. 2004). In the next ten years, it is reasonable to expect genetic, biochemical and cellular insights into the functions of most transporters in pollen, and their integration with pollen development in *Arabidopsis* and in other higher plants.

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Small GTPases and Spatiotemporal Regulation of Pollen Tube Growth

Jae-Ung Hwang · Zhenbiao Yang (✉)

Department of Botany and Plant Sciences and Center for Plant Cell Biology, Institute for Integrative Genome Biology, University of California, Riverside, CA 91521, USA
zhenbiao.yang@ucr.edu

Abstract During in vivo growth, pollen tubes make a long journey toward the ovule, responding to long- and short-distance guidance cues and elongating through different female tissues. Thus, pollen tube growth and guidance require complicated inter- and intracellular signaling, integration of multiple signals, and spatiotemporal coordination of the downstream responses necessary for targeted exocytosis. ROP, a plant-unique family of Rho small G proteins, is known to function as a versatile molecular switch in a variety of processes such as cell morphogenesis, stress and defense responses, hormonal responses, and directional growth of pollen tubes and root hairs. Current evidence suggests that ROP GTPase controls pollen tube growth temporally and spatially, coordinating multiple downstream signaling pathways. This chapter will review up-to-date findings about ROP GTPase signaling in pollen tubes and will discuss how ROP regulates pollen tube growth.

1 Introduction

Rho-family small GTPases are pivotal signaling switches conserved in eukaryotic organisms. By cycling between inactive GDP-bound and active GTP-bound forms, Rho GTPases participate in signaling to cytoskeletal organization and dynamics and membrane trafficking, and the list of cellular processes regulated by these GTPases keeps increasing (Burridge and Wennerberg 2004; Gu et al. 2004). Examples of processes controlled by Rho GTPase signaling include cell polarity development, polar cell growth, cell morphogenesis, cell migration, and cell division.

An important feature of Rho family GTPases is their capacity to act at key nodes of a signaling network, because of their ability to integrate multiple upstream signals, to coordinate multiple downstream pathways, and to participate in feedback regulation. This feature lies in the large number of functional partners that can physically associate with the small GTPases. Four classes of Rho-interacting proteins are known to act as upstream regulators. Docking/scaffolding proteins target Rho GTPases to specific membranes or membrane domains. Guanine nucleotide exchange factors (GEFs)

activate membrane-associated GTPases by replacing GDP with GTP. GTPase-activating proteins (GAPs) promote GTP hydrolysis leading to the inactivation of GTPases, whereas guanine nucleotide-dissociation inhibitors (GDIs) inhibit the activation of GTPases by suppressing nucleotide exchange and sequestering GTPases in the cytosol. Any of these regulators is capable of perceiving an upstream signaling, giving Rho GTPases the ability to integrate multiple signals. A single Rho GTPase is capable of activating multiple functionally distinct effector proteins, achieving the coordination of multiple downstream pathways.

Another key feature of Rho GTPases is their functional diversity due to their ability to orchestrate a wide range of intracellular signaling networks. In humans, at least 20 genes encode Rho family proteins, which are roughly classified into five functionally distinct subfamilies: Rho, Rac, Cdc42, Rnd, and RhoBTB (Burridge and Wennerberg 2004). Similarly, their interacting partners are usually encoded by gene families with functionally distinct members. For example, two structurally unrelated classes of Rho GEFs have been described in humans. Classic Dbl homology–Pleckstrin homology (DH–PH) domains containing Rho GEF proteins form the largest family of RhoGEF in humans, composed of 69 homologs (Erickson and Cerione 2004; Rossman et al. 2005). The divergent functional motifs flanking the DH–PH domain indicate that these GEFs are capable of linking Rho to diverse extracellular stimuli and intracellular pathways. In addition, another family of GEFs (DOCK180-related RhoGEFs) has emerged (Brugnera et al. 2002; Meller et al. 2005). Regardless of the structural dissimilarity, DOCK180-related RhoGEFs activate Rho G proteins probably via a similar mechanism of GDP–GTP exchange as shown in SopE of *Salmonella typhimurium* (Erickson and Cerione 2004). The presence of numerous Rho GEF molecules emphasizes the capability of Rho proteins as versatile molecular switches.

Interestingly, plants possess a single subfamily of Rho-like GTPases, named ROP (Rho-like GTPase from plants) (Yang and Watson 1993; Winge et al. 2000; Zheng and Yang 2000; Vernoud et al. 2003). The *Arabidopsis* genome encodes 11 different ROP genes, which can be classified into four subgroups based on amino acid sequence homology (Zheng and Yang 2000). Because no apparent orthologs of mammalian Rho, Rac, or Cdc42 have been reported, ROP appears to mediate diverse cellular responses as the sole family of Rho GTPase in plants. Studies using gain of function and loss of function approaches have revealed that ROPs have many physiological roles such as control of pollen tubes and root hair growth, cell shaping, stress response, defense to pathogen attack, and hormonal signaling (Gu et al. 2004). The functional diversity and specificity of Rop GTPases in plants can be accounted for by different isoforms of ROPs as well as by the diversity of their interacting proteins (Table 1).

Table 1 Expression of ROPs and upstream regulators and effectors in mature *Arabidopsis* pollen. The expression of ROPs, upstream regulators GEF, GDI, and GAP, and effector RIC, are indicated. Information from several independent studies is summarized (sources shown in References). "Present" indicates that the transcript of a gene is present in mature pollens. "Abundant" and "selective" stand for high expression and preferential expression in mature pollens compared to other vegetative tissues, respectively. If the presence of transcript, either abundant or not, was reported by at least one study, the gene is considered to be expressed in mature pollens. "Not determined" indicates that the gene's expression has never been tested. Three of 11 RICs have not been given AGI numbers (RIC8, 9, and 11)

Gene name		AGI no.	Expression in pollen	Refs.
ROP	ROP1	At3g51300	Abundant	[1, 4–6]
	ROP2	At1g20090	Absent	[1, 4–6]
	ROP3	At2g17800	Abundant	[1, 4–6]
	ROP4	At1g75840	Absent	[1, 4–6]
	ROP5	At4g35950	Abundant	[1, 2, 4–6]
	ROP6	At4g35020	Absent	[1, 2, 4–6]
	ROP7	At5g45790	Absent	[4–6]
	ROP8	At2g44690	Present	[3–6]
	ROP9	At4g28950	Present	[3–6]
	ROP10	At3g48040	Present	[3–6]
	ROP11	At5g62880	Present	[3–5, 7]
GEF	RopGEF1	At4g38430	Present	[5, 6, 8]
	RopGEF2	At1g01700	Absent	[5, 6, 8]
	RopGEF3	At4g00460	Absent	[5, 6, 8]
	RopGEF4	At2g45890	Absent	[6, 8]
	RopGEF5	At5g05940	Absent	[5, 8]
	RopGEF6	At3g55860	Absent	[8]
	RopGEF7	At5g02010	Absent	[5, 6, 8]
	RopGEF8	At3g24620	Abundant and selective	[5, 6, 8]
	RopGEF9	At4g13240	Abundant and selective	[5, 6, 8]
	RopGEF10	At5g19580	Abundant	[5, 6, 8]
	RopGEF11	At1g52240	Abundant and selective	[5, 6, 8]
	RopGEF12	At1g79880	Abundant	[6, 8]
	RopGEF13	At3g16130	Abundant and selective	[6, 8]
	RopGEF14	At1g31650	Present	[5, 6, 8]
	SPK1	At4g16340	Absent	[5, 6]
GDI	RhoGDI1	At3g07880	Abundant	[5–7]
	RhoGDI2	At1g12070	Abundant	[5–7]
	RhoGDI3	At1g62450	Abundant and selective	[5–7]

Table 1 (continued)

Gene name		AGI no.	Expression in pollen	Refs.
GAP	RopGAP1	At5g22400	Present	[5–7]
	RopGAP2	At4g03100	Absent	[5–7]
	RopGAP3	At2g46710	Present	[5–7]
	RopGAP4	At3g11490	Absent	[5, 6]
	RopGAP5	At1g08340	Absent	[5–7]
	RopGAP6	At2g27440	Absent	[5–7]
	RopGAPx	At5g61530	Abundant	[5, 6]
RIC	RIC1	At2g33460	Abundant	[5, 6, 9]
	RIC2	At1g27380	Present	[5, 9]
	RIC3	At1g04450	Abundant	[9]
	RIC4	At5g16490	Present	[5, 6, 9]
	RIC5	At3g23380	Abundant	[9]
	RIC6	At2g20430	Abundant	[5, 6, 9]
	RIC7	At4g28560	Abundant	[6, 9]
	RIC8		Not determined	
	RIC9		Present	[9]
	RIC10	At4g04900	Present	[6, 9]
	RIC11		Not determined	

1. Li et al. (1998); 2. Kost et al. (1999); 3. Gu et al. (2003); 4. Cheung et al. (2003); 5. Pina et al. (2005); 6. Honys and Twell (2004); 7. Wu G, Hwang JU, Yang Z, unpublished; 8. Gu Y, Li S, Lord EM, Yang Z, unpublished; 9. Wu et al. (2001)

2 ROP GTPases and Pollen Tube Growth

Gene expression analysis using RT-PCR, promoter-GUS assay, and microarrays has revealed that up to seven ROP genes are expressed in mature *Arabidopsis* pollen (Table 1). Three members of group IV (*ROP1*, *ROP3*, and *ROP5*) are highly expressed in mature pollen grains (Li et al. 1998; Kost et al. 1999; Honey and Twell 2004; Pina et al. 2005). ROP8 of group I and all three members of group II (ROP9, ROP10, and ROP11) are also expressed in mature pollen (Gu et al. 2003; Cheung et al. 2003). ROP1, ROP3, and ROP5 share a high level of amino acid sequence identity (> 86%), and thus they are expected to be functionally redundant in the control of pollen tube tip growth (Li et al. 1999; Gu et al. 2004). Indeed, ROP1 and ROP5 displayed similar cellular localization and caused similar growth depolarization when overexpressed in pollen tubes (Li et al. 1999; Kost et al. 1999; Wu et al. 2001). In the following sections of this chapter, we will refer to the members of

subgroup IV collectively as "ROP1" for simplicity. However, there is still the possibility that they play subtle distinct in vivo functions during pollen tube development. A study from maize pollen supports this possibility. In maize pollen, only a subset of ROPs (*ROP2*, *8*, and *9*) are highly expressed (Christensen et al. 2003). ROP2 and ROP9 share higher identity in amino acid sequence (> 97%), even in untranslated regions, and yet knocking out one of the two nearly identical ROP genes produced minor but significant transmission defects via male gametophytes without any obvious cellular defect (Arthur et al. 2003).

The importance of ROPs in pollen tube growth was first implicated by a study showing that pea *Rop1Ps*, the first known plant Rho GTPase (Yang and Watson 1993), is preferentially expressed in pollen and that Rop1Ps is localized to the apical region of the plasma membrane in pollen tubes (Lin et al. 1996). In yeast and animal cell systems, Rho such as Rac and Cdc42 play an essential role in cell polarity establishment and maintenance, primarily through their role in the regulation of the actin cytoskeleton (Burridge and Wennerberg 2004). Subsequent studies accumulated evidence supporting the hypothesis that ROP regulates both temporal and spatial aspects of pollen tube growth. Inhibition of ROP activity by microinjection of anti-Rop1Ps antibody into pea pollen tube arrested tip growth without affecting cytoplasmic streaming (Lin and Yang 1997). Overexpression of dominant negative forms (T20N, D121A) of *Arabidopsis* ROP1 inhibited the tip growth of *Arabidopsis* or tobacco pollen tubes (Li et al. 1999; Fu et al. 2001). Dominant negative forms of ROP1 are expected to block endogenous ROP1 signaling by sequestering upstream activators (GEFs). Antisense *ROP1* expression also brought about pollen tube growth inhibition in *Arabidopsis* (Li et al. 1999). The growth retardation caused by ROP1 inactivation indicates that the activity of ROP1 and/or its closely related ROPs is essential for tip growth. Overexpression of DN-rop5 mutant also blocked pollen tube growth in tobacco (Kost et al. 1999). As mentioned above, it is likely that ROP1, ROP3, and ROP5 have a redundant cellular function in their control of pollen tube growth. However, the relative contribution of each of these ROPs in pollen tube growth regulation needs to be evaluated using individual and multiple knockout mutants.

When GFP-ROP1 was expressed in *Arabidopsis* pollen, the pollen with low green fluorescent protein (GFP) fluorescence (low level of ROP1 expression and small increase of ROP1 activation) displayed better germination and tube growth (Gu et al. 2003). This observation further supports the role of ROP1 in the control of pollen tube growth. Interestingly, pollen tubes with strong GFP fluorescence (higher level of ROP1 expression and excess ROP1 activation) produced bulbous tubes, resulting from the depolarization of pollen tube growth (Li et al. 1999; Gu et al. 2003). Expression of constitutively active (CA) forms of ROP1 (G15V or Q64L) caused much more severe depolarization of pollen tube growth (Li et al. 1999; Gu et al. 2003). The isotropic growth

(bulbous pollen tube formation), rather than polar growth, induced by excess ROP1 activation suggests the importance for the control of ROP1 activity of the spatial regulation of tip growth in pollen tubes. This observation also implies a role for ROP1 in the control of pollen tube growth polarity, and thus the directional control of pollen tube growth (see the chapter by Malhó, this volume).

Orthologs of ROP1 appear to be present in monocot species, supporting a common ROP-dependent mechanism for the control of pollen tube growth in plants. Rice and maize possess *ROP* genes closely related to *Arabidopsis ROP1* (*OsRacB* and *OsRacD*, and *ZmROP2*, 4, and 9) (Christensen et al. 2003). Recent studies support the view that these ROPs may be the functional orthologs of ROP1 in the control of pollen tube growth in monocots, by showing that maize *ROP2* and *ROP9* are highly expressed in pollen and that loss-of-function mutants of maize *ROP2* displayed transmission defects in the male gametophyte (Arthur et al. 2003).

Unlike ROP1, the function of other ROPs expressed in pollen, i.e., ROP8 to ROP11, remains mysterious but appears to be distinct from that of ROP1. Overexpression of ROP8, 9, 10, or 11 did not induce severe depolarization of pollen tube growth as ROP1 and ROP5 did (Gu et al. 2005; Hwang et al. 2005). The cellular localization tested with GFP-fused forms of these ROPs was not confined to the tip apex like GFP-ROP1 (Cheung et al. 2003; Vernoud et al. unpublished data). In addition, the expression of these ROPs did not alter ROP1 signaling significantly in the pollen tube apex (Gu et al. 2005; Hwang et al. 2005). The recruitment of a ROP1 downstream effector, RIC4 (ROP-interactive CRIB motif-containing protein 4), to the apical plasma membrane was not affected significantly by overexpression of these ROPs, whereas ROP1 overexpression did have an effect (Gu et al. 2005; Hwang et al. 2005). Thus, ROP8–11 may play an in vivo role yet to be identified but clearly distinct from that of ROP1.

3 Tip-Localized Active ROP1 Couples the Spatial and Temporal Control of Pollen Tube Elongation

The preferential localization of ROP1 to the apex and the effect of ROP1 activation on both tip growth and growth polarity suggest that localized ROP1 activation in the tip is not only critical for growth control, but may also define growth polarity or the site for tip growth. A recent investigation of in vivo ROP1 activity using a live GFP-based ROP1 activity marker supports this hypothesis. An active ROP1 reporter was developed based on RIC4 (Hwang et al. 2005). RIC4 is a downstream effector molecule of ROP1 (Wu et al. 2001; Gu et al. 2005). It preferentially binds to the GTP-bound active ROP1 both in vitro and in vivo (Gu et al. 2005). A mutant of RIC4, in which C-terminal 21-

amino acids behind the CRIB motif were deleted (RIC4ΔC), retained the full capacity of RIC4 to specifically bind to the active form of ROP1 but did not alter pollen tube growth when transiently expressed in tobacco pollen tubes (Hwang et al. 2005). Visualization of GFP-RIC4ΔC reveals that active ROP1 forms a tip-high gradient in the extreme of the pollen tube plasma membrane (PM), termed the apical cap of active ROP1, which apparently corresponds

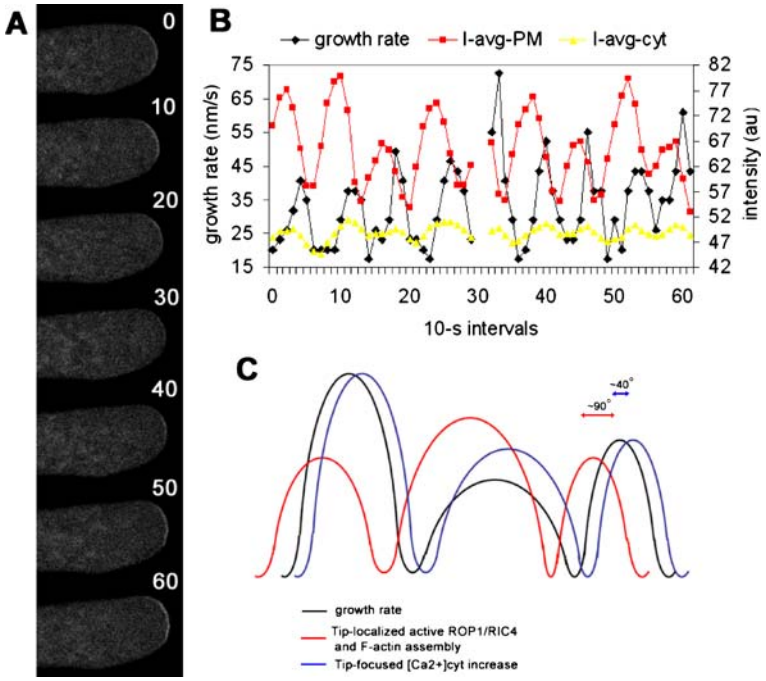

Fig. 1 The oscillatory tip-localized ROP1 activity coordinates two downstream pathways, $[Ca^{2+}]_c$ dynamics and F-actin assembly. **a** A typical round of tip-localized ROP1 oscillation of ~ 60–70 s period. In vivo ROP1 activity was visualized by GFP-RIC4ΔC and the average intensity of GFP-RIC4ΔC localized to the tip apical PM oscillates with the tip growth rate. Numbers at the *top right* indicate the elapsed time (seconds) from the beginning (0 s). **b** Quantitative data showing that a tip-localized ROP1 activity increase leads the burst of tip growth, supporting the control of tip growth by active ROP1. The graph was reproduced from Hwang et al. (2005) with permission of MBC (This needs to be obtained). The average activity of ROP1 in the tip apical PM was quantified by measuring the average intensity of GFP-RIC4ΔC localized to the tip PM (I-avg-PM). I-avg-PM oscillates 10–20 s ahead of tip growth rate, whereas the amount of GFP-RIC4ΔC in the tip (I-avg-c) remains relatively constant with only minor fluctuations. **c** A cartoon showing the phase relations between tip growth, tip-localized ROP1 (RIC4) activity, F-actin assembly, and $[Ca^{2+}]_c$ increase. Tip-localized ROP1 activity oscillation is on average ~ 90° ahead of tip growth. Two downstream targets of active ROP1 appear to be activated differentially: F-actin assembly is stimulated early, which is in similar phase with ROP1 activation, while the $[Ca^{2+}]_c$ increase is stimulated late (~ 40° behind tip growth)

to the active growth domain (Fig. 1a). The tip-localized ROP1 activity is very dynamic in normal growing pollen tubes and was found to oscillate with the same frequency as the growth rate (Hwang et al. 2005; Fig. 1). Tip-localized ROP1 activity starts increasing about 10–20 s ahead of the tip growth rate increase in a 70-s period of oscillation (i.e., 90° ahead of growth). This phase relationship is different from oscillations of ion fluxes such as Ca^{2+}, Cl^{-}, K^{+}, and H^{+}, which appear to be associated with the growth or post-growth events (Holdaway-Clarke et al. 1997; Messerli et al. 1999, 2000). The temporal relation between ROP1 activation and growth thus supports a crucial role of active ROP1 in pollen tubes (Fig. 1c).

The formation of the apical cap of active ROP1 at the presumed active growth site indicates that ROP1 activation may be spatially regulated to de-

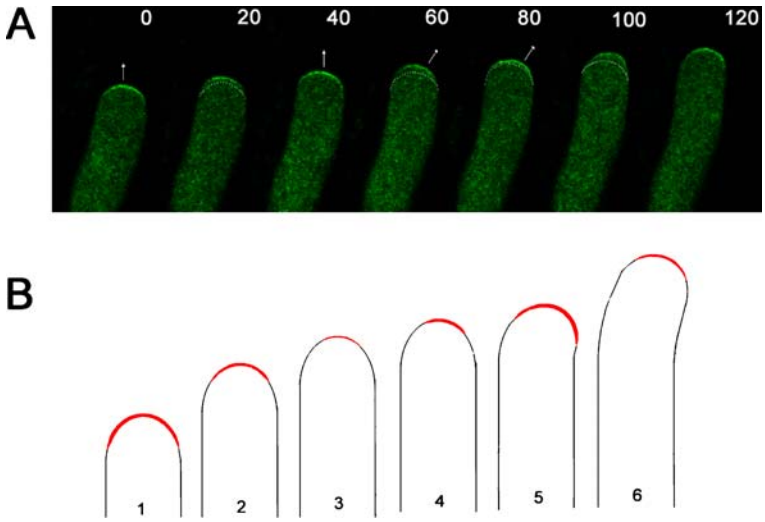

Fig. 2 The tip-localized ROP1 activation defines the growth polarity. **a** Representative pollen tube images with tip-localized ROP1 activity. The pollen tube grew in a rapid oscillatory mode of 40–60-s period. Numbers at the *top right* indicate the elapsed time (seconds) from the beginning (0 s). The repositioning of the active ROP1 apical cap is indicated by *arrows*, which point in the future growth direction. The position of the tip apex at the previous time point is outlined with *white dots*. **b** A cartoon showing the spatiotemporal regulation of tip growth by tip-localized ROP1. The *red lines* indicate the amount and distribution of active ROP1 in the tip apical PM. One round of oscillation is divided into six phases (1–6): (1) tip-localized active ROP1 at the peak; (2) growth burst with tip active ROP1 amount being decreased; (3) interpulse state of ROP1 oscillation with growth rate being decreased; ROP1 activity starts increasing again from the minimum level; (4) ROP1 activity increases, pointing in new growth direction; the growth rate is in the minimum; (5) the new growth polarity is stabilized with increased ROP1 activity; ROP1 activity is in the maximum with growth rate being increased slowly; and (6) tip growth bursts toward new growth direction defined by active ROP1

fine the region of the plasma membrane for growth. This notion is supported by a tight correlation between the distribution of GFP-RIC4ΔC in the apical region of the plasma membrane and spatial changes in pollen tube growth. In pollen tubes overexpressing ROP1 or RIC4, an increase in the size of the GFP-RIC4/RIC4ΔC-containing apical cap was associated with an increase in tube width (Wu et al. 2001; Gu et al. 2005; G Wu et al. unpublished data). Conversely, a reduction of tube width was correlated with a decrease in the size of the apical cap in tubes expressing ROP negative regulators (G Wu et al. unpublished data). In reorienting pollen tubes, the GFP-RIC4ΔC apical cap was relocated to the future growth site before observable growth direction occurred (Hwang et al. 2005). Tobacco pollen tubes treated with 0.5 nM latrunculin B (LatB) displayed transient growth retardation and then resumed the oscillatory growth in a new direction (Hwang et al. 2005). The apical cap of GFP-RIC4ΔC relocation toward a future growth direction was detected clearly before the growth surge (Fig. 2). Consistent with the effects of ROP1 inactivation and overactivation on pollen tube growth, these observations strongly support the hypothesis that the spatiotemporal dynamics of tip-localized ROP activation couples the spatial and temporal regulation of tip growth (Fig. 2b). The apical cap of ROP1 activity can thus be considered as a dynamic growth organizer in time and space, which predicts the timing of the next growth surge and the position or direction of new growth. Given that Rho GTPases have been established as an integrator of different upstream signals, it is reasonable to speculate that ROP1 can integrate various signals from female tissues that regulate and guide pollen tube growth as the pollen tube is targeted to the ovule for fertilization (see the chapter by Johnson and Lord, this volume). Therefore, it is not surprising that the study of pollen tube signaling has been focused on two most interesting questions: (1) how the spatiotemporal dynamics of ROP1 activity is regulated and (2) how the localized ROP1 activity signals to localized growth.

4 Tip-Localized ROP1 Controls Pollen Tube Growth by Coordinating Multiple Pathways

Pollen tubes need to coordinate several cellular activities necessary for localized growth, including the production and targeting of secretory vesicles, fusion of these vesicles to the target site, and remodeling of cell walls (see the chapters by Malhó and by Geitmann and Steer, this volume). We postulate that ROP1 signaling can coordinate various pathways that, in turn, regulate these cellular activities. Recent studies provide strong evidence that support such a hypothesis (Kost 1999; Li et al. 1999; Wu et al. 2001; Gu et al. 2005). Perhaps the most insightful study is the identifica-

tion of putative ROP target proteins, named RICs (ROP-interactive CRIB-motif-containing proteins). The *Arabidopsis* genome encodes 11 RICs, which share the ROP-binding CRIB motif but are highly diverse in their primary structures.

4.1 RIC3-mediated Formation of the Tip Focused $[Ca^{2+}]_c$ Gradient

Growing pollen tubes form a steep gradient of cytosolic free calcium ($[Ca^{2+}]_c$) in the apex (see the chapter by Hepler et al., this volume). Blocking of the $[Ca^{2+}]_c$ gradient formation abolished tip growth (Pierson et al. 1994; Malhó et al. 1995), and localized $[Ca^{2+}]_c$ increase induced by releasing Ca^{2+} ionophores or photolysis of caged Ca^{2+} caused growth reorientation to the direction of $[Ca^{2+}]_c$ increase (Malhó and Trewavas 1996). Thus, the tip-focused Ca^{2+} gradient is not only required for tip growth but also appears to be involved in the control of polar growth. How is the tip-focused $[Ca^{2+}]_c$ gradient established?

Several lines of evidence support the notion that ROP1 mediates the establishment of the tip $[Ca^{2+}]_c$ gradient. Inhibition of ROP signaling by microinjection of ROP-specific antiserum completely abolished the tip-focused $[Ca^{2+}]_c$ gradient, causing immediate growth inhibition (Li et al. 1999). *Arabidopsis* pollen tubes overexpressing *ROP1* or antisense *ROP1* gene displayed altered sensitivity of pollen tube growth to external Ca^{2+} concentrations (Li et al. 1999; Gu et al. 2005). In root hairs, it has been shown that ROP overexpression induced growth inhibition and delocalized the tip-focused $[Ca^{2+}]_c$ gradient (Molendijk et al. 2001). In *Agapanthus umbellatus* pollen tubes, Camacho and Malhó (2003) also reported a tight interaction between ROP function and the $[Ca^{2+}]_c$ gradient in the control of apical secretion and membrane recycling. Using caged analogs of GTP and antisense oligonucleotides to reduce ROP expression, Camacho and Malhó (2003) obtained data that led them to suggest that Ca^{2+} and ROPs act differentially but in a concerted form in the sequential regulation of pollen tube secretion and membrane retrieval. The most recent experiment that links ROP1 to Ca^{2+} is the functional study of RIC3 (Gu et al. 2005), a ROP1 target in pollen tubes.

RIC3 is one of the 11 *Arabidopsis* RICs. RIC3 interacted specifically with the active form of ROP1 in both in vitro pull down and in vivo fluorescence resonance energy transfer (FRET) assays (Gu et al. 2005). It induced the depolarization of tip growth as ROP1 did, and its action is dependent upon ROP1 activation (Wu et al. 2001; Gu et al. 2005). RIC3 overexpression increased cytosolic Ca^{2+} levels and induced an extended $[Ca^{2+}]_c$ gradient. RIC3 appears to regulate the influx of extracellular Ca^{2+} because the putative Ca^{2+} channel blocker, La^{3+}, suppressed the RIC3 overexpression phenotype (Gu et al. 2005). Furthermore, RIC3 overexpression or loss-of-function mutant pollen tubes displayed altered sensitivity to extracellular (ext) Ca^{2+}. RIC3-overexpressing

Arabidopsis pollen tubes grew well in the environment of low $[Ca^{2+}]_{ext}$, whereas wild-type pollen tube growth was generally suppressed under such conditions. In contrast, RIC3 loss-of-function mutant pollen tubes needed a higher $[Ca^{2+}]_{ext}$ to achieve growth (Gu et al. 2005). Since RIC3 is a novel protein, the mechanism by which RIC3 promotes the influx of extracellular Ca^{2+} remains unknown.

4.2 RIC4-Dependent Accumulation of Tip-Localized F-actin

The actin cytoskeleton in pollen tubes can be said to be composed of two major subpopulations: the extensive longitudinal actin cables, which appear to be responsible for the reverse fountain pattern of cytoplasmic streaming, and the dynamic F-actin structures in the tip (see the chapter by Yokota and Shimmen, this volume). There is controversy over the detailed description of the dynamic F-actin structure in the tip, depending on the kind of F-actin markers and cell preservation methods applied (Fu et al. 2001; chapters by Hepler et al. and by Yokota and Shimmen, this volume), but it is generally believed that the dynamic F-actin structure in the tip is more directly related to the control of secretory vesicle targeting and fusion. The selective disruption of tip-localized dynamic F-actin with low concentration of actin-depolymerizing drugs (e.g., cytochalasin D and latrunculin A/B) inhibited pollen tube growth without disrupting major cytoplasmic streaming (Gibbon et al. 1999; Vidali et al. 2001). It has been shown that the dynamic tip F-actin assembly is affected by active ROP1. Overexpressed ROP1 promoted the formation of short actin filaments in the tip apex, whereas inhibition of ROP1 by expressing DN-rop1 or RopGAP1 and GDI1 led to the extension of longitudinal actin cables to the tube tip (Fu et al. 2001). Similar results were reported with a tobacco ROP1 homolog (NtRac1). NtRac1 overexpression promoted the formation of a dense meshwork of actin filaments in pollen tube tips (Chen et al. 2003).

In mammalian cells, Rho stimulates F-actin formation primarily by activating actin nucleating factors or via Rho kinases, which in turn activates actin stabilizing factors or inactivates actin depolymerizing factors (ADFs). However, it is not well understood how ROP regulates F-actin dynamics in plant systems. Recently, RIC4 was proposed to mediate ROP1-induced F-actin assembly (Gu et al. 2005). RIC4 specifically interacts with the active form of ROP1 and is localized to the tip apical plasma membrane in an active ROP1-dependent way (Gu et al. 2005). RIC4 overexpression enhanced the formation of tip-localized F-actin as ROP1 did, but did not increase $[Ca^{2+}]_c$ in pollen tubes (Fu et al. 2001; Gu et al. 2005). RIC4-induced depolarized tip growth could be suppressed by increasing actin depolymerization with a treatment of LatB or coexpression of profilin. *Arabidopsis* pollen tubes overexpressing RIC4 showed the enhanced accumu-

lation of tip F-actin and displayed increased resistance to LatB-induced growth inhibition, whereas loss-of-function mutants were hypersensitive to LatB (Gu et al. 2005). In animal cells, the Arp2/3 complex is pivotal for actin polymerization, primarily associated with Cdc42/Rac. *Arabidopsis* possesses homologs of subunits of the Arp2/3 complex, but genetic disruption of Arp2/3 complex subunits barely affected pollen tube growth (Li et al. 2003). This suggests that ROP-mediated F-actin assembly in pollen tubes differs from Cdc42/Rac-mediated actin assembly in animal and yeast cells. The mechanism by which RIC4 regulates actin organization remains to be determined.

4.3 ROP and Phosphoinositide Signaling

Phosphoinositides (PIs) are important signaling molecules, either serving as substrates for the production of the inositol-1,4,5-trisphosphate (IP3) second messenger or directly binding to a signaling protein. Emerging evidence supports a role for PIs in pollen tube growth (Monteiro et al. 2005; chapter by Žárský et al., this volume). Interestingly, it was shown that recombinant ROP5 was associated with a phosphatidylinositol phosphate (PIP) kinase activity from tobacco pollen tubes (Kost et al. 1999). Using a GFP-tagged PH domain, which specifically binds PIP2, the product of PIPK, it was shown that PIP2 was localized to the apical region of the pollen tube plasma membrane (Kost et al. 1999). Blocking PIP2 by a specific PH domain also inhibited pollen tube growth. These results suggest that ROP1 and PIP2 signaling may converge for control of pollen tube growth. Although it is unclear how PIPK and its product PIP2 are implicated in pollen tube growth, both of F-actin assembly and $[Ca^{2+}]_c$ accumulation are potential targets (see the chapter by Žárský et al., this volume).

4.4 ROP Regulation of Actin Depolymerization Factors (ADFs)

In addition to Arp2/3 complex-mediated nucleation, animal Rho is known to regulate several actin binding proteins (ABPs) such as ADF/cofilin, filamin, and formin (Maekawa et al. 1999; Vadlamudi et al. 2002). The involvement of ADFs in ROP1-mediated control of F-actin dynamics was reported in tobacco pollen (Chen et al. 2003). Tobacco pollen-specific ADF (NtADF1) suppressed the NtRac1-induced depolarization of tip growth and F-actin assembly. The nonphosphorylatable mutant ADFs with an Ala substitution at the Ser-6 position were much stronger, whereas a phospho-mimicking mutant with Ser-to-Asp change was not effective in suppressing NtRac1 phenotype development (Chen et al. 2003). Interestingly, NtRac1 overexpression increased the phosphorylation of NtADF1 and presumably its inactivation, suggesting

that ROP regulates the phosphorylation status of ADF and thereby controls the dynamics of F-actin in the pollen tube (Chen et al. 2003). In maize, it was reported that ADF is phosphorylated by a Ca^{2+}-dependent protein kinase activity (Allwood et al. 2001). Whether ROP regulates the phosphorylation status of ADF and whether it is Ca^{2+} dependent are questions now requiring an answer.

4.5 Other Possible Downstream Pathways: Formins, Exocyst, and RabA4

In animal cells, the actin nucleating process by formins is the rate-limiting step for de novo actin filament synthesis, and some formins are known to be activated by interaction with Rho via a G protein-binding domain (Evangelista et al. 2003). It is reported that a group I formin (AFH1) of 20 *Arabidopsis* formin family members promoted pollen tube growth at a low level of expression and induced depolarization of tip growth at a high level of expression (Cheung and Wu 2004; Michelot et al. 2005). This suggests that actin nucleation via AFH1 is involved in pollen tube growth. However, it is unclear whether formins are involved in ROP-mediated F-actin assembly, because plant formins lack the G protein-binding domain.

For polar cell growth, directional vesicle delivery, targeted fusion, and secretion are critical. Polar exocytosis most likely requires F-actin reorganization and Ca^{2+} increases. In addition to F-actin formation and Ca^{2+} increase, Rho is known to regulate the exocytosis via other mechanisms. One potent candidate of ROP effector for targeted exocytosis is exocyst. Exocyst is a conserved eight-subunit protein complex, which is localized to sites of polarized exocytosis and required for vesicle targeting and docking to specific plasma membrane domains (Roumanie et al. 2005; chapter by Žárský et al., this volume). In yeast cells, one of eight subunits, Sec3, is known to interact directly with GTP-bound Rho1 and functional Rho1 is required both to establish and to maintain polarized Sec3 localization (Guo et al. 2001). Sec3 is not the only target of Rho to control exocyst. Another subunit, Exo70, interacts with Rho3 independently from Sec3 (Roumaine et al. 2005). The *Arabidopsis* genome contains homologs of all eight subunits of exocyst complex (Elias et al. 2003; Cole et al. 2005). Particularly, pollen of Sec8 knockout mutants showed transmission defects, and some of Sec3 and Exo70 coding genes are highly expressed in pollen (Cole et al. 2005). It will thus be interesting to investigate a putative link between exocyst and ROP1.

Another potential linkage is the family of RAB small G proteins. *Arabidopsis* RabA4 proteins and tobacco Rab11 (homologs of animal Rab11) are reported to play a critical role in polarized vesicle delivery and exocytosis in polar growing cells (de Graaf et al. 2005; Preuss et al. 2004). Both localize at the pollen tube tip (de Graaf et al. 2005; YJ Lee et al. unpublished data) and mutations on NtRab11 caused defects in tip growth and tip F-actin structure

in tobacco pollen tubes (de Graaf et al. 2005). In *Arabidopsis* trichoblasts, the recruitment of ROP to the growth initiation site was dependent on Rab function, because brefeldin A inhibited the polar localization of ROPs (Molendijik et al. 2001). Thus, crosstalk between ROP and RAB small G proteins for coordinating targeted secretion is quite likely.

5
Check and Balance Between RIC3 and RIC4 Pathways: Roles in Actin Dynamics

As discussed above, active ROP1 stimulates two important downstream pathways, the assembly of tip-localized F-actin and the tip-focused $[Ca^{2+}]_c$ gradient, via two distinct mediators, RIC3 and RIC4 (Gu et al. 2005) (see Fig. 3). The assembly of tip-localized F-actin could provide forces for targeted delivery and accumulation of secretory vesicles to the future growth site; too much and less dynamic F-actin could be the physical barrier to vesicle fusion (Giner et al. 2005). Cytosolic Ca^{2+} is also required for vesicle fusion, but high levels of cytosolic Ca^{2+} disrupt F-actin (see the chapter by Yokota and Shimmen, this volume). Efficient tip growth thus requires a critical balance between Ca^{2+} and dynamic F-actin. Not surprisingly, RIC3-dependent Ca^{2+} and RIC4-dependent F-actin pathways counteract each other (Gu et al. 2005). RIC4-overexpressing pollen tubes showed enhanced F-actin assembly but a less pronounced tip-focused Ca^{2+} gradient. In contrast, RIC3-overexpressing tubes developed higher tip $[Ca^{2+}]_c$ but completely lost the tip fine F-actin structure. RIC4 coexpression suppressed RIC3-induced depolarized growth in an F-actin dependent manner, and LatB treatment blocked the suppression of RIC3 signaling by RIC4 coexpression. RIC3 coexpression suppressed RIC4-induced depolarized growth in a Ca^{2+}-dependent manner, and blocking of Ca^{2+} influx by La^{3+} inhibited the counteracting effect of RIC3 on RIC4 signaling (Gu et al. 2005). Clearly, control mechanisms are needed to check the activation of these two pathways and make them balanced for optimum growth. It was found that when RIC3 and RIC4 were expressed at 1 : 2 molecular ratio in tobacco pollen tubes, the apical morphology was comparable to the control tubes and a normal tip F-actin structure was restored (Gu et al. 2005). Interestingly, *ric3* and *ric4* double loss-of-function *Arabidopsis* pollen tubes grew with normal apical morphology (although shorter than wild-type tubes), whereas single loss-of-function mutants displayed retarded growth (Gu et al. 2005). In the RNAi *ric3* and *ric4* double mutant, cells could still express diminute amounts of RIC3 and RIC4, thus justifying normal morphology but shorter growth.

How do the RIC3- and RIC4-dependent pathways counteract each other? An increase of cytosolic $[Ca^{2+}]$ activates several actin binding proteins (ABPs) (see chapter by Yokota and Shimmen, this volume, for detailed dis-

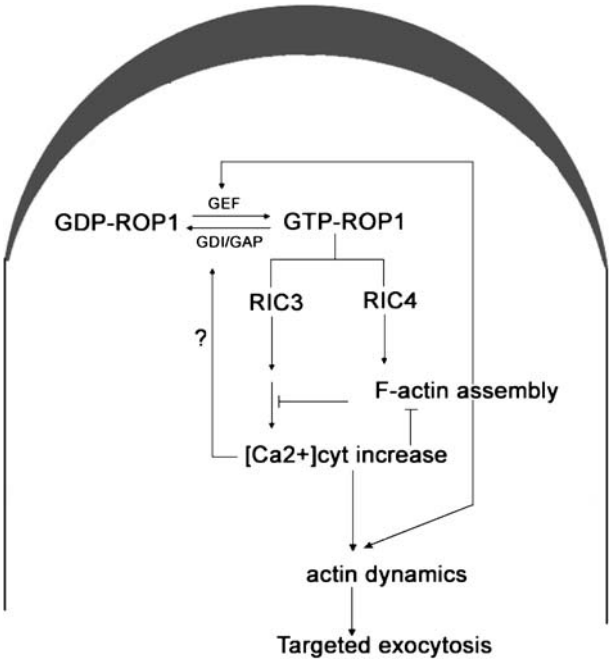

Fig. 3 A model for a ROP signaling network regulating pollen tube growth. Active ROP1 stimulates F-actin assembly and $[Ca^{2+}]_c$ increase via RIC4- and RIC3-dependent pathways. F-actin assembly may control the targeted accumulation of secretory vesicles, and late $[Ca^{2+}]_c$ increase induces actin depolymerization, promoting vesicle fusion. By regulating targeted vesicle delivery and fusion, tip-localized active ROP1 couples the temporal and spatial aspects of tip growth. F-actin assembly feed-forwardly promotes the accumulation of active ROP1 to the tip, forming a positive feedback loop of signal amplification. A $[Ca^{2+}]_c$ increase terminates the active ROP1 amplification probably by counteracting the F-actin pathway. It remains to be answered whether tip high Ca^{2+} directly activates the downregulation of ROP1 activity via, or independently of, F-actin

cussion). For example, in tobacco pollen tubes, profilin and RIC3 completely restored the RIC4 overexpression phenotype (Gu et al. 2005).

Ca^{2+} channels at the pollen tube plasma membrane are also putative targets for F-actin to suppress a $[Ca^{2+}]_c$ increase. RIC3-induced Ca^{2+} increase is dependent on the influx of extracellular Ca^{2+} (Gu et al. 2005), and recent studies of Ca^{2+} channel activities in pollen protoplasts provide some hints to explain why increased F-actin assembly by RIC4 suppressed RIC3-dependent Ca^{2+} accumulation in the tip. Wang et al. (2004) have shown that the voltage-operated inward Ca^{2+} channel activity found in the plasma membrane of pollen protoplasts was stimulated by actin depolymerization. Increased tip-localized F-actin assembly by RIC4 may affect the activities of these Ca^{2+} influx channels in the tip plasma membrane, thereby inhibiting the increase of $[Ca^{2+}]_c$.

6
ROP1 Temporally Coordinates Ca^{2+} and F-actin

How are RIC3- and RIC4-dependent pathways balanced to promote rapid tip growth? A temporal lag between RIC4 activation of the actin assembly and the RIC3 activation of Ca^{2+} accumulation may underscore the mechanism that coordinates these two important signaling pathways. In normal growing pollen tubes, tip-localized ROP1 activity (RIC4 activity), tip-localized F-actin accumulation, and tip-focused $[Ca^{2+}]_c$ seem to oscillate with the same frequency as tip growth oscillation (Messerli et al. 2000; Fu et al. 2001; Hwang et al. 2005). The correlation test based on frequency (period length) indicates that RIC4-dependent F-actin accumulation is stimulated earlier than RIC3-dependent $[Ca^{2+}]_c$ increase (Fig. 2c). The lag of the RIC3 activation of $[Ca^{2+}]_c$ increase could result from a more lengthy multistep signaling for this pathway, although the details have yet to be worked out.

The tip-localized ROP1 activity/RIC4 localization to the tip peaks approximately 90° ahead of growth rate (Hwang et al. 2005). The RIC4-dependent assembly of tip-localized F-actin oscillates in the same phase with or slightly lagging the tip-localized ROP1 activity, but leading tip growth (Fu et al. 2001; Hwang et al. 2005). In contrast, the RIC3-modulated tip-focused $[Ca^{2+}]_c$ gradient was found to oscillate slightly behind growth. Temporally differential activation of the RIC3-dependent Ca^{2+} pathway and RIC4-dependent F-actin pathway by active ROP1 and the counteraction between the two pathways may enable the rapid oscillatory tip growth and tip-localized ROP1 activation (Gu et al. 2005; Hwang et al. 2005). Increase in F-actin assembly immediately follows the increase in the amount of active ROP1 and may subsequently modulate vesicle transport. At the same time, cortical F-actin assembly may lead to inhibition of Ca^{2+} influx, thus preventing premature $[Ca^{2+}]_c$ increase from interfering with polarity stabilization via F-actin. Subsequently, the delayed RIC3-dependent $[Ca^{2+}]_c$ increase could promote F-actin depolymerization via Ca^{2+}-sensitive ABPs, causing a growth burst toward the determined growth polarity.

6.1
How is ROP1 Activity Regulated?

As discussed above, in vitro cultured pollen tubes display dynamic spatiotemporal changes in ROP1 activity. In vitro pollen tube growth does not require exogenous growth stimuli, so the dynamics of ROP1 activity in these tubes must be achieved by a self-organizing mechanism. In migrating animal cells and budding yeast cells, random initiation of local activation of Rho GTPases is stabilized by positive feedback amplification (Weiner et al. 2002; Wedlich-Soldner et al. 2003). In yeast, Cdc42-mediated positive feedback activation involves the regulation of Cdc24 (Cdc42 GEF) by both actin (a target of Cdc42

signaling)-dependent and -independent mechanisms (Wedlich-Soldner et al. 2003; Wedlich-Soldner and Li 2003). In animal cells, PIP_3 and Rac accumulate to the leading edge of migrating cells and are amplified via a positive feedback loop that requires PI3K and Rac GTPase (Weiner et al. 2002). Similar mechanisms may be implicated in the regulation of the apical cap of active ROP1 in growing pollen tubes (Hwang et al. 2005). Polymerization of F-actin in the apex appears to participate in the positive feedback regulation of ROP1 activity. Increased F-actin assembly by RIC4 overexpression induced sustained increase in tip-localized ROP1 activity, whereas LatB treatment did the opposite (Hwang et al. 2005). To maintain the tip-localized ROP activity, negative regulation is also required to confine ROP activity to the apex. Otherwise, ROP1 would be excessively activated, resulting in the delocalization of active ROP1 and depolarization of tip growth. Furthermore, in normal tubes undergoing growth oscillation, a negative feedback mechanism must be required to downregulate ROP1 activity once it reaches a peak. A detailed molecular basis for the feedback mechanisms is thus required.

During pollination, tip-localized ROP activity is most likely regulated by guidance signals so that pollen tubes can properly navigate toward the ovule. It is not understood how ROP activation is regulated by spatial cues. Female reproductive tissues are known to release guidance cues (see the chapters by Johnson and Lord and by Guermonprez et al., this volume). Receptors in the pollen tube plasma membrane are speculated to perceive the spatial cues and convey the signal into the cytoplasm, which may finally trigger ROP activation. Plants possess two types of unconventional GEFs, RhoGEFs and SPIKE1, but lack the classic Dbl homology domain containing GEF. RopGEFs have been recently identified to have GEF activity toward ROPs, and have a plant-unique guanine nucleotide exchange domain (Berken et al. 2005; G Ying et al. unpublished data). However, it is not understood if and how RopGEF is involved in ROP activation by intrinsic or extrinsic stimuli.

Recent studies suggest the possibility that pollen receptor-like protein kinases (RLKs) may link guidance cues to ROP signaling by recruiting RopGEF. RopGEF is also known as a kinase partner protein (KPP) in tomato (Kaothien et al. 2005). KPP is a pollen-specific protein and interacts with the cytoplasmic domain of receptor-like kinases (LePRK1 and LePRK2). KPP was phosphorylated in tomato pollen, and induced depolarization of pollen tube growth and abnormal F-actin structure like ROP overexpression did (Kaothien et al. 2005). It is also known that ROP may form a complex with RLKs like CLAVATA1 (Trotochaud et al. 1999). These results support an exciting hypothesis that ROP activity is regulated by RopGEF, which is regulated by its interaction with or phosphorylation by membrane receptor kinases. In mature *Arabidopsis* pollens, up to eight members of RopGEF are expressed (Table 1). When transiently overexpressed in tobacco pollen tubes, these RopGEFs induced depolarization of tip growth (Y Gu et al. unpublished data). In rice, a heterotrimeric G protein was shown to function upstream of the small GT-

Pase OsRac1 in the early steps of signaling (Suharsono et al. 2002), and such proteins have been implicated in the regulation of pollen tube growth (Ma et al. 1999). Future studies should address whether RopGEF is regulated by receptor-like kinases and/or heterotrimeric G protein-coupled receptors.

7 Perspectives

Active ROP1 is localized to the tip apical plasma membrane and oscillates with tip growth, stimulating downstream pathways in a temporally coordinated way, e.g., an early RIC4-dependent F-actin pathway and a delayed RIC3-dependent Ca^{2+} pathway. Tip-localized active ROP1 forms a tip-high gradient (apical cap), which corresponds to the area where secretory vesicles accumulate. Active ROP1 defines the active growth domain probably by spatially regulating the vesicle delivery and fusion. Thus, tip-localized dynamic ROP1 activity couples the temporal and spatial aspects of the tip growth. Since the first plant Rho was identified in pea pollen, considerable progress has been made in understanding the functional roles that ROP plays in pollen tube growth. Major future challenges will be to unravel how the spatial cues regulate ROPs in the pollen tube tip, which may involve signal perception via specific receptors, transduction to ROP activation, and/or inactivation via GEF, GDI, or GAP. The mechanisms by which active ROP1 stimulates the downstream pathways such as F-actin assembly and $[Ca^{2+}]_c$ increase also need to be characterized. In addition to RIC3 and RIC4, future studies may reveal the new ROP1 effectors that may be needed for ROP1 to coordinate other downstream pathways such as RabA4 and exocyst components. Investigation of a potential interaction of the ROP1 signaling network with other mechanisms in the control of pollen tube growth should provide a full picture of the molecular mechanisms underlying pollen tube growth and guidance.

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Lipid Metabolism, Compartmentalization and Signalling in the Regulation of Pollen Tube Growth

V. Žárský[1,2] (✉) · M. Potocký[1] · F. Baluška[3] · F. Cvrčková[2]

[1]Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, 160 00 Prague 6, Czech Republic
zarsky@ueb.cas.cz

[2]Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague 2, Czech Republic
zarsky@ueb.cas.cz

[3]Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Abstract To understand the biological context of lipid metabolism and signalling in pollen, we have to consider male gametophytes as organisms optimised for their role in sexual reproduction, but also for survival in dry conditions. While our knowledge of molecular mechanisms governing pollen development and pollen tube growth is based on the studies of a few model species (mostly Arabidopsis, tobacco, petunia and lily), important aspects of pollen development may vary substantially among species. Moreover, current understanding of pollen lipid biochemistry is rather fragmentary, since biochemically tractable amounts of pollen material are difficult to obtain, and knowledge of sporophytic lipid metabolism and signalling cannot be simply transferred to the study of male gametophytes.

1 Introduction

Lipid signalling is crucial for the organization of membrane domains and membrane trafficking. Conserved lipid-binding domains, such as PH, FYVE, PX, PHD, ENTH, and C2 target relevant proteins towards specific lipid-enriched membrane domains (van Leeuwen et al. 2005). Phosphorylated derivatives of the phospholipid phosphatidylinositol (phosphoinositides) translate the complex "language" of lipid signalling into membrane traffic (Czech 2003; Downes et al. 2005; Roth 2004), assembly of specific membrane domains (Carlton and Cullen 2005), and signal-triggered reorganization of the actin cytoskeleton (Huang et al. 2003; Yin and Janmey 2003; Downes et al. 2005). These interactions modulate activities of actin-binding proteins (Huang et al. 2003), which have an immediate impact on the actin cytoskeleton (Downes et al. 2005; Yin and Janmey 2003). Pollen tubes provide an excellent model object for the study of these aspects, since their extremely polarized tip growth requires a complex molecular machinery integrating

both the secretory pathway and dynamic actin structures regulated by lipid signalling.

2
Pollen-specific Features of Lipid Metabolism

2.1
Pollen Tubes Use Aerobic Ethanolic Fermentation to Support Lipid Biosynthesis

Pollen tubes grown aerobically in vitro in sucrose media with extremely high respiratory rate produce copious amounts of ethanol (Bucher et al. 1995). It has been suggested that this energy metabolism pathway may provide a competitive advantage in areas of low oxygen pressure within the style (Gass et al. 2005).

More than half of the carbohydrate flows through the fermentative pathway; the flux towards ethanol is regulated by sugar availability rather than by oxygen (Tadege et al. 1999). Products of aerobic fermentation – acetaldehyde and ethanol – are further metabolised in a pathway that involves pyruvate decarboxylase (PDC), alcoholdehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS), but bypasses mitochondrial pyruvate dehydrogenase, similar to situation in yeast (Tadege et al. 1999; Mellema et al. 2002). PDC and ADH are among the major pollen proteins. Interestingly, maize mutants lacking ADH produce viable pollen, since ADH+/adh– heterozygotes segregate in a Mendelian fashion (Freeling and Bennett 1985), while a petunia mutant impaired in the pollen-specific PDC2 locus produces pollen unable to outcompete wild-type pollen in mixed pollination/fertilization experiments. The genetically proven non-essentiality of ADH suggests that this enzyme may act mainly to prevent accidental accumulation of reactive acetaldehyde during pollen tube growth, while the main flux leads via PDC and ALDH towards high molecular weight compounds, especially lipids (Gass et al. 2005).

2.2
Triacylglycerols, Surface/Exine Lipids and Oleosins in Pollen-pistil Recognition

The variability of Angiosperm species with respect to the contents of storage compounds (starch or lipids) in mature, post-anthesis pollen grains have been known since the 1950s (Baker and Baker 1979). Prominent lipid accumulation in mature pollen correlates with insect pollination, while starch-accumulating species are often pollinated by wind. Major interspecific diversity with respect to metabolic flux directions and timing has to be therefore expected. However, it is reasonable to assume that this diversity will affect mostly triacylglycerol storage and mobilization, rather than membrane lipid metabolism, transport and signalling.

Intracellular triacylglycerols are stored in oil bodies, in complex with phospholipids and amphipatic structural proteins – oleosins. Oleosins form large gene families, with 16 different proteins in *Arabidopsis*. Apart from oleosins expressed in pollen grains (Kim et al. 2002), there are paralogues expressed in tapetal cells and involved in oil bodies development during tapetum maturation and disintegration. During anther dehiscence, these proteins accumulate inside cavities of the exine; at pollination, they are released to the stigma surface. In *Arabidopsis*, oleosins and lipases comprise more than 90% of pollen coat proteins, suggesting their crucial function in pollen-stigma recognition interaction (Mayfield et al. 2001). Rapid evolution of pollen oleosins in *Arabidopsis* and closely related species further supports this hypothesis (Schein et al. 2004).

The functional importance of surface pollen lipids was first documented by conditional pollen sterility of cuticle-deficient Arabidopsis *cer6* mutant that may be rescued by high humidity (Preuss et al. 1993). Mutant pollen lacks very-long-chain fatty acids (VLCFA); cloning of the *CER6/CUT1* locus showed that the mutated protein resembles fatty acid-condensing enzymes (Fiebig et al. 2000). Disruption of the locus CER10 encoding enoyl-CoA reductase also compromises VLCFA synthesis, resulting in a general cell expansion and endocytosis defect (Zheng et al. 2005). VLCFA are also essential components of the stigmatic surface needed for the pollen tube penetration (Wolters-Arts et al. 1998).

3
Membrane Lipids and Phospholipids in Pollen Tubes

3.1
Membrane Lipid Metabolism

There are very few biochemical studies addressing specifically membrane lipid metabolism in pollen tubes. In the first detailed analysis of the dynamics of phospholipid metabolism in elongating lily pollen tubes, Helsper et al. (1986b) observed considerable incorporation of [^{32}P] orthophosphate into phospholipids, mainly phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP), phosphatidylglycerol (PG) and phosphatidic acid (PA). As the total amount of lipid-bound phosphorus did not change during germination, continuous incorporation of labelled phosphorus indicated high phospholipid turnover. Radioactively marked *myo*-inositol also exhibited high turnover; it was incorporated not only into phospholipids, but also in pectic polysaccharides. The dynamics of phospholipids was shown to be linked to the activity of lipases. Activities of phospholipase C (PLC) and phospholipase D (PLD) in pollen tubes and the presence of their products diacylglycerol (DAG), Ins(1,4,5)P_3

and PA have been documented (Helsper et al. 1986, 1986b, 1987). Dorne et al. (1988) found that in *Nicotiana sylvestris* the total amount of lipids does not increase during pollen germination and tube growth, despite membrane surface expansion and continuous incorporation of radioactively labelled acetate into all lipid classes (dominated by neutral lipids – triacyl glycerols and sterols). In fact, the total phospholipid content decreased after an initial increase preceding germination. All these approaches suggest a rapid turnover of membrane components in growing pollen tubes.

3.2 Phospholipid Signalling and Metabolism – Phospholipase C and Inositol Trisphosphate

Polyphosphate inositol in the form of phytic acid was recognized as an important phosphate-rich storage compound accumulated in the mature pollen (Jackson and Lisnkens 1982). Molecular analysis of *Arabidopsis* inositol polyphosphate kinases (IPK) uncovered two genes known as AtIPK2α and β lacking, in contrast to some animal orthologues, the CAM binding domain implying Ca^{2+}-independent regulation (Xu et al. 2005). Antisense transgenic plants with reduced amounts of AtIPK2α transcripts showed increased pollen germination and pollen tube growth as compared to the wild type, especially at suboptimal Ca^{2+} concentrations; other phenotypes of these plants suggest an important role of inositol polyphosphate metabolism in Ca^{2+} signalling, possibly via modification of inositol trisphosphate activity (Xu et al. 2005). Alkaline phytase, catalysing hydrolysis of phytic acid and activated by Ca^{2+}, was recently characterized from pollen (Jog et al. 2005).

Based on the known central role of Ca^{2+} tip-focused gradient in pollen tube growth, as well as knowledge of Ca^{2+}-related PI signalling from animal systems, early work in membrane-related pollen signalling was centered on the expected role of PLC activity in phosphatidylinositol 4,5 bisphosphate (PIP_2) cleavage and DAG and Ins(1,4,5)P_3 production. Franklin-Tong et al. (1996) showed that the Ca^{2+} dynamics is correlated with Ins(1,4,5)P_3 in poppy pollen tubes. However, in this particular study extraordinary high concentrations of mastoparan (25 μM) were used to stimulate Ins(1,4,5)P_3 production. Our unpublished data (Zarsky and Obermeyer) show that at this concentration mastoparan causes membrane permeabilization, making the interpretation of data very difficult. Further experiments with caged Ins(1,4,5)P_3 and other drugs (Franklin-Tong et al. 1996; Malhó 1998) showed clearly that Ins(1,4,5)P_3 is involved in $[Ca^{2+}]_c$ dynamics and appears to participate in the reorientation of tube growth.

Small GTPases are well-known regulators of cell polarity; members of the Rop family are localized to the growing pollen tube tip and participate in the regulation of the Ca^{2+} gradient (Li et al. 1999; Hwang and Yang, this volume). Furthermore, they were shown to interact at the pollen tube tip

with the phosphatidylinositol monophosphate kinase (PI-PK) whose product, PIP_2, accumulates in the Rop-containing tip-focused membrane domain (Kost et al. 1999). Overexpression of the PIP_2-binding domain of PLC inhibited, as predicted, pollen tube elongation. Rop GTPases may control local activity of PI-PK in the tip of growing pollen tubes, and PIP_2 might serve as a substrate for the PLC producing Ins(1,4,5)P_3. The abundant monomeric G-actin-binding protein profilin may also be involved in the PIP_2-dependent regulation of actin cytoskeleton and signalling activities, including those mediated by the Ca^{2+} gradient (Clarke et al. 1998). There are two functionally distinct classes of profilin isoforms in plants, differing in their affinity for PIP_2 (Yokota and Shimmen, this volume). Von Witsch et al. (1998) observed membrane localization of profilin in microspores and pollen. The role of PIP_2 was further re-examined by Monteiro et al. (2005) who showed that photorelease of both caged PIP_2 and Ins(1,4,5)P_3 modified growth and caused reorientation of the growth axis. However, measurements of cytosolic free calcium ($[Ca^{2+}]_c$) and apical secretion revealed significant differences between effects of PIP_2 or Ins(1,4,5)P_3. Release of PIP_2 led to transient growth arrest, increase of cytosolic Ca^{2+}, and inhibition of apical secretion. By contrast, a concentration of Ins(1,4,5)P_3 which caused a $[Ca^{2+}]_c$ transient of similar magnitude, stimulated apical secretion and caused severe growth perturbation. Therefore, there may be different targets of these two signalling intermediates.

The *Arabidopsis* genome encodes nine PI-PLC isoforms, with three of the proteins being most probably inactive. Plant PI-PLCs do not have PH domain, similar to animal PI-PLCζ (Hunt et al. 2004). Partially purified PI-PLCs from different plant species are Ca^{2+}-dependent in their activities; e.g. AtPLC2 preferentially hydrolyses PIP_2 and maximal activity in vitro is achieved at 1 μM $[Ca^{2+}]_c$ (Otterhag et al. 2001). Systematic analysis of expression and Ca^{2+} sensitivity of Arabidopsis PI-PLC isoforms revealed overlapping but specific expression and Ca^{2+} optimum ranging from 1 μM for AtPLC2,4 and 5 to 3 μM for AtPLC1 and 3. AtPLC4 is highly expressed in mature and developing Arabidopsis pollen (Hunt et al. 2004). This particular PLC isoform was the only one that retained in vitro activity (20%) even in the presence of 2 mM EGTA (Hunt et al. 2004). High levels of AtPLC4 accumulation in mature dehydrated pollen may assist early Ins(1,4,5)P_3-induced Ca^{2+} release during pollen germination. Tip-localization of AtPLC4-GUS fusion in growing pollen tubes implies function in tip growth and fertilization (Hunt et al. 2004). Since animal spermatic PLC triggers $[Ca^{2+}]_c$ oscillations within mouse egg, leading to egg activation (Saunders et al. 2002); it is tempting to speculate that similar function might be linked to pollen PI-PLC during final stages of embryosac double-fertilization (Hunt et al. 2004). Pan et al. (2005) described PI-PLC activity and cloned two PLC isoforms, LdPLC1 and LdPLC2, from *Lillium daviddi* pollen. PI-PLC activity in pollen protoplasts was enhanced by exogenous calmodulin and G protein agonist cholera toxin, and

decreased by G protein antagonist, pertussis toxin. Application of the PI-PLC inhibitor U-73122 abolished the cholera toxin-mediated stimulation of PLC activity and led to the decrease of $[Ca^{2+}]_c$ in pollen grains, further indicating the presence of PIP_2-PLC- Ins(1,4,5)P_3-Ca^{2+} cascade in pollen.

3.3
Phospholipid Signalling – Phospholipase D and Phosphatidic Acid

Alongside with phosphoinositides, other (phospho)lipids such as PA have been shown to control cell polarity and vesicular trafficking in yeast and animal cells (for plants see Testerink and Munnik 2005). PLD activity and accumulation of its product PA was observed already in the early pollen phospholipids analyses (Dorne et al. 1988; Helsper et al. 1986b). Although PLDs activities in vegetative plant cells in respect to stress signalling and adaptation have been intensively studied (see e.g., Testerink and Munnik 2005), less attention has been paid to their role in housekeeping cellular functions. The analysis of PLD functions in plants is complicated by the overlapping activities of different PLD isoforms in the same cell (for plant PLD family phylogenetic analysis see Elias et al. 2002). PLDζ1 was localized to the clear zone vesicles of root hairs; its inducible overexpression caused branching of root hairs while PLD inhibition resulted in loss of root hairs (Ohashi et al. 2003). This reveals that PLD and PA are essential signalling molecules driving tip-growth of root hairs, suggesting their role in other tip-growing cells as well.

PA can be produced in plant cells either by cleavage of PIP_2 by PLC followed by phosphorylation of DAG by DAG kinase (connecting thus phosphoinositide and PA signalling), or directly by PLD-mediated hydrolysis of structural phospholipids such as PC and PE. During hydrolysis, PLD covalently binds to the phosphatidyl group and then transfers it to a nucleophile. The nucleophile is usually water, but in the presence of 1-butanol, stable and biologically inactive phosphatidylbutanol is formed preferentially. Thus, incubation of plant cells in 0.2% to 0.5% butanol inhibits PA production. Using this approach, Potocky et al. (2003) showed that PLD-mediated production of PA is crucial for the tip growth of tobacco pollen tubes. PLD inhibition in vivo by 0.25% 1-butanol led to rapid arrest of pollen tube growth, whereas application of PA-containing liposomes increased the growth rates. The dynamics of BODIPY-labelled PA internalization and pollen tube endomembrane localization was followed (Fig. 1). Distinct PLD activities with specific PIP_2 and Ca^{2+} requirements and with different dynamics were detected during pollen germination and tube elongation suggesting multiple roles of PA in signalling and membrane domain formation (Potocky et al. 2003). Pollen tube growth inhibition caused by 1-butanol was partly relieved by taxol, supporting the hypothesis that some plant PLDs may function as a MAP in the regulation of MT dynamics (Gardiner et al. 2001; Potocky et al. 2003). PLD inhibition rapidly disturbs the dynamics of pollen tube endomembrane system,

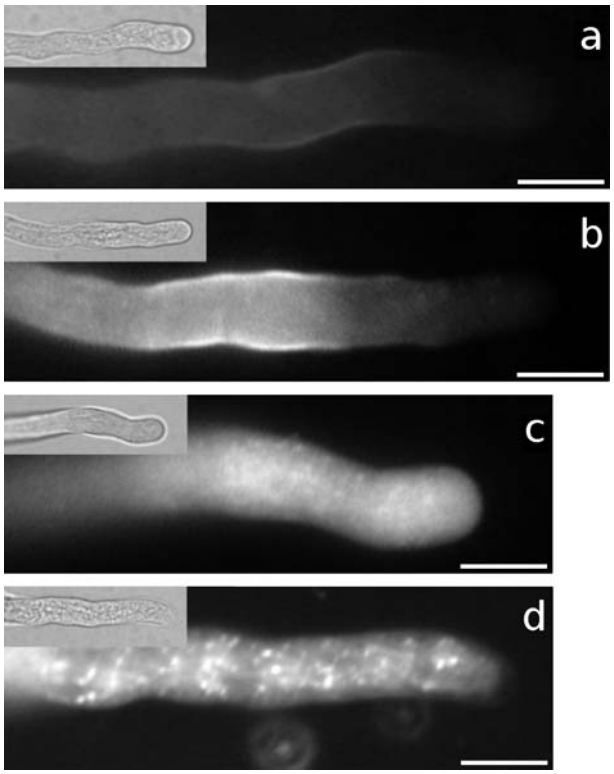

Fig. 1 Cellular distribution of the BODIPY-PA in growing pollen tubes of tobacco. Incorporation of 0.5 μM BODIPY-PA after 5 min (**a**), 30 min (**b**), 60 min (**c**), and 90 min (**d**) is shown. Bar = 10 mm. Adapted from Potocky et al. 2003

as visualised by FM1-43 dye. Recovery of pollen tube elongation by external addition of PA liposomes is correlated also with the recovery of tip membrane dynamics (Potocky et al. 2004). A similar disturbance of tip localized secretory machinery by 1-ButOH treatment was observed also in *Agapanthus umbellatus* pollen tubes, resulting in pollen tube growth arrest, disappearance of tip-focused calcium gradient, profound rearrangement (bundling) of F-actin microfilaments, apex expansion and disappearance of secretory vesicles (Monteiro et al. 2005). Although we have repeatedly observed fragmentation of long F-actin filaments under comparable conditions (Potocky et al., in preparation), data of both groups point to the profound effect of PLD activity on the pollen tube cytoskeleton.

Our understanding of the regulatory function of PA in the plant cell F-actin dynamics was recently augmented by the finding that PA added to *Arabidopsis* cell suspension cultures as well as to poppy pollen leads to a significant increase in F-actin levels, a process mediated by the heterodimeric capping protein from *Arabidopsis* (AtCP; Chris Staiger, pers. comm). AtCP binds not

only to PA, but also to PIP_2 with a similar affinity. However, the intracellular concentration of PA is orders of magnitude higher, making it the major binding partner of AtCP. Interaction of PA with AtCP inhibits the actin-binding activity of AtCP, rendering it unable to block the barbed end of actin filaments allowing rapid filament assembly from an actin monomer pool that is buffered with profilin (Chris Staiger, pers. comm).

Stress-induced activation of multiple phospholipid pathways was also documented in growing pollen tubes (Zonia and Munnik 2004). Although PLD activity is constitutive during tube growth, hypo-osmotic treatment rapidly increases PLD-derived PA content and hyper-osmotic stress is characterised by elevated levels of phosphatidylinositol bisphosphates. Specific patterns of membrane modifications in response to osmotic stress were recognized – hypoosmotic treatment stimulated pollen tube expansion assisted by the stimulation of PLD activity and 7-fold accumulation of PA. In contrast, hyperosmotic treatment (50 to 400 mM NaCl) caused pollen tube shrinking, inhibited PLD activity, reduced PA levels and induced an increase in PIP_2 isomers (Zonia and Munnik 2004). This work also indicates that the majority of PA in pollen tubes is produced via PLD pathway and not PLC/DAG kinase pathway. Interpretation of the data with hyperosmotic treatment in this study is unfortunately compromised by the fact that NaCl at the concentration used exhibits general toxic effects (like KCl and/or LiCl). Inhibition of pollen tube growth imposed by 100 mM NaCl can be completely restored by the addition of 40 mM $CaCl_2$ – i.e., even further increase in osmotic potential of the in vitro growth medium (Brink 1924). Further analysis of this topic is thus required.

Despite the developmental dehydration before anthesis, the possible role of ABA signalling in pollen development is unclear (e.g., Rose et al. 1996). *Arabidopsis* transcriptome analysis suggests two ABA related genes relatively overexpressed in pollen (Pina et al. 2005) and the ABI1 PP2C protein phosphatase, a negative regulator of ABA response, was recently discovered as a binding partner of PA. Three closely related PP2C isoforms are expressed in tricellular and mature *Arabidopsis* pollen, including ABI1 mRNA itself (At3g11410; At1g72770; At4g26080). It is therefore highly probable that PA in developing pollen might intervene also in putative signalling pathways regulated by PP2C phosphatases activities (Zhang et al. 2005).

4
Lipid Signalling in the Cellular and Whole-plant Context

4.1
Expression Analysis of the Phosphoinositide Pathway in Pollen

Recent studies reported unique characteristics of the pollen transcriptome emphasizing a functional skew of pollen transcriptome toward vesicle traf-

ficking, cytoskeleton and signalling (Twell, Oh and Honys, this volume). Using publicly available expression data, we attempted a more detailed analysis of the expression of genes putatively involved in generation and transduction of PPI signalling. We selected 176 genes, from which 158 were found on Affymetrix ATH1 chip. These included phospholipases, PI and PIP kinases, phosphoinositide phosphatases, inositol polyphosphate kinases and phosphatases together with proteins containing known phosphoinositide binding modules such as PH, PX, FYVE and ENTH domains (Fig. 2, Table 1, see also http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). Interestingly, 89 genes (58.4%) were called "present in the pollen", which is nearly twice as much compared to whole pollen transcriptome, where only 29% of genes are called present in pollen (Pina et al. 2005). Strong presence of PPI signalling related genes in pollen is also evident from the number of pollen-exclusive and pollen-enriched genes (7.0% and 26.8% respectively). In comparison, there are no PPI related genes selective for leaves and only 1–3.4% are enriched in this tissue (depending on the dataset). Same conclusion can be drawn from roots (data not shown). Overall analysis also indicates that PPI signalling is equally important in all stages of pollen development, as from 116 PPI genes called present during pollen development, 45 are expressed preferentially during unicellular and bicellular stage and 54 genes show maximum expression in tricellular stage and in mature pollen.

If we look closely at specific gene families, the most striking feature is the absence of Vps34 transcript, coding for type III PI3-kinase (Fig. 3). Vps34 is the only PI3-kinase identified so far in *Arabidopsis*. Although Vps34 is absent in pollen, other components of PI3P signalling such as FYVE domain and PX domain containing proteins and Fab1 (PI3P-5 kinase) orthologues are present in pollen (see below) and $PI(3,5)P_2$ can be detected during growth of tobacco pollen tubes (Zonia and Munnik 2004). It is difficult to accept absence or substantial down-regulation of PI3P in developing pollen and pollen tubes, as it seems to be an important specific component of plant endosomes (Samaj et al. 2005). The genes putatively involved in PIP_2 pathway have generally the strongest signal in pollen (Fig. 2) and often contain novel sequence features, further pointing to the central role of this phosphoinositide in pollen.

Both type II and type III PI4 kinases are expressed in pollen with type II being the dominant subfamily, including some of the most expressed pollen loci (PI4Kγ3, PI4Kγ4, PI4Kγ8, Fig. 2). Type II PI4 kinases, albeit yet not functionally characterised in plants, are supposed to be insensitive to wortmannin, well-known inihibitor of PI3- and type III PI4-kinases. The expression profile of PI-kinase isotypes yielded conclusions similar to that of PI-4 kinases; both PIP kinases synthetising PIP_2, $PI(3,4)P_2$ (type I/II), and $PI(3,5)P_2$ (type III) are expressed in pollen. Again, the pollen-specific and most expressed isoform PIPK10 belongs to plant specific subfamily A, and

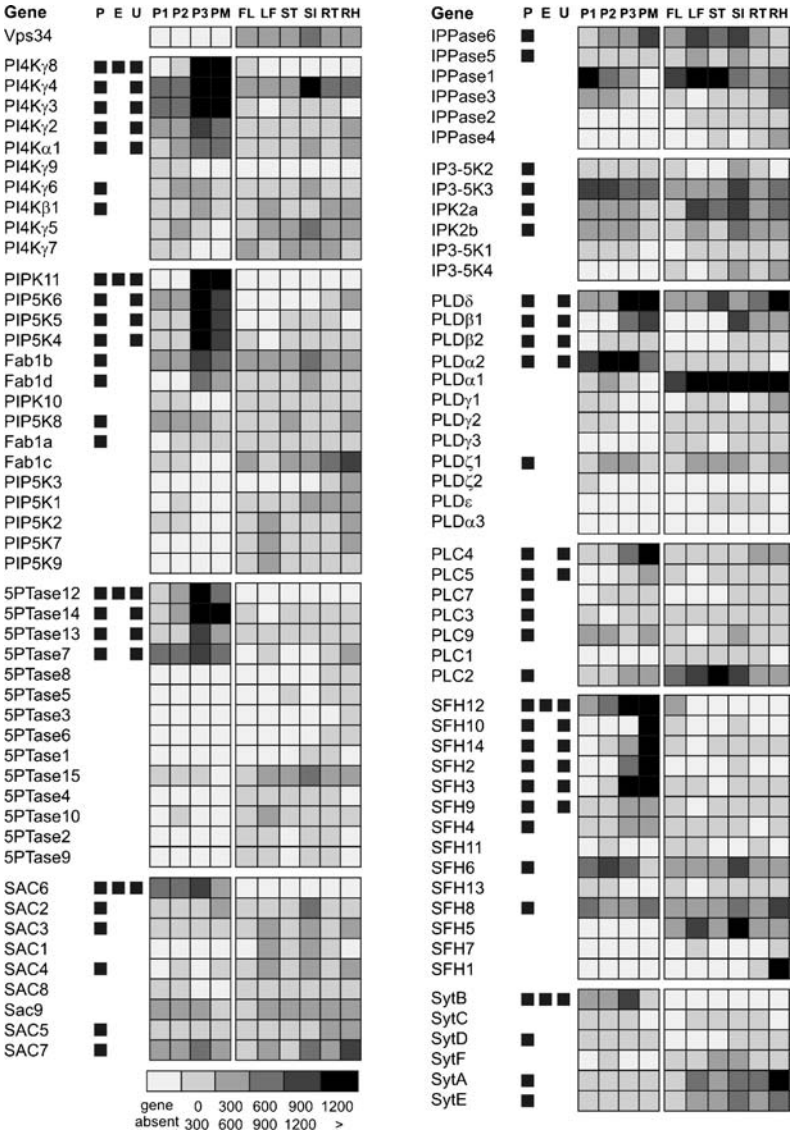

Fig. 2 Expression analysis of selected phosphoinositide pathway genes from Arabidopsis. Expression data for haploid male gametophyte development was extracted from the works of Honys and Twell (2005) together with selected data from sporophytic tissues that have been subject to normalization and statistical analysis. Also selected data from Pina et al. (2005) and AtGenExpress database (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) were renormalized and included into analysis. For additional information, see (http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). P, gene present in pollen; E, gene exclusive in pollen; U, gene upregulated in pollen; P1, uninucleate microspores; P2, bicellular pollen; P3, immature tricellular pollen; PM, mature pollen grains; FL, flowers; LF, leaves; ST, stems; SI, siliques; RT, roots; RH, root hairs

Table 1 Expression analysis of *Arabidopsis* genes with phosphoinositide binding domains. Expression data for haploid male gametophyte development was extracted from the works of Honys and Twell (2004) together with selected data from sporophytic tissues which has been subject to normalization and statistical analysis. Also selected data from Pina et al. (2005) and the AtGenExpress database (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) were renormalized and included into analysis. For additional information, see (http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). P, gene present in pollen; E, gene exclusive in pollen; U, gene upregulated in pollen; S, expression signal in pollen; FC, fold change

Gene	AGI code	P	E	U	S	FC	Gene	AGI code	P	E	U	S	FC
PH domain containing proteins													
ArfGap1	At1g1087						PLDzeta2	At3g0563					
ArfGap2	At1g6086	*			147		PH8	At1g7320	*			219	
ArfGap3	At5g6198	*		*	729	14.1	Praf1	At1g6592					
ArfGap4	At5g1330						Praf2	At3g4766	*	*	*	346	6.9
ADL6	At1g1029	*			241		Praf4	At1g7695					
ADL3	At1g5961	*			267		Praf5	At5g4214					
PH2	At2g2970	*			154		Praf6	At3g2327	*		*	339	16.0
PH3	At5g0571	*			298		Praf7	At4g1437	*	*	*	141	9.6
PH4	At1g7773	*	*	*	637	19.7	Praf8	At5g1235					
OBP1	At2g3103						Praf9	At5g1942					
OBP2	At4g0818	*		*	598	4.2	PH9	At4g3278					
OBP3	At1g1317						PH10	At3g6330					
OBP4	At4g1246						PH11	At5g4387	*			131	
OBP5	At4g2254	*			169		PH12	At3g2281					
START1	At3g5480	*		*	1035	107	PH14	At4g1667					
START2	At4g1904	*		*	934	2.8	PH15	At4g1735	*			181	
START3	At5g4556						PH16	At5g4744					
START4	At5g3518	*			609		PDK11	At3g1054	*		*	811	5.8
START5	At2g2832	*		*	866	3.0	PDK12	At5g0451					
RhoGAP1	At4g2458	*		*	826	13.9	Vps34	At1g6049					
RhoGAP2	At5g1215	*			124		PI4Ka1	At1g4934	*		*	621	2.8
PH5	At1g4809	*			355		PI4Kb1	At5g6407	*			238	
PLDzeta1	At3g1678	*			240								
FYVE domain containing proteins													
Praf1	At1g6592						Praf9	At5g1942					
Praf2	At3g4766	*	*	*	346	6.9	Fab1a	At4g3324	*			233	
Praf4	At1g7695						Fab1b	At3g1427	*			868	
Praf5	At5g4214						Fyve1	At1g2011	*			558	
Praf6	At3g2327	*		*	339	16.0	Fyve2	At3g4323	*			343	
Praf7	At4g1437	*	*	*	141	9.6	Fyve4	At1g6169	*			172	
Praf8	At5g1235						RingFyve	At1g6162	*			547	
PX domain containing proteins													
PX-PXA1	At2g1590						PX6	At5g5844	*			92	
PX3	At2g2535						PLDzeta1	At3g1678	*			240	
PX-SPEC	At4g3216	*			350		PLDzeta2	At3g0563					
PX4	At5g0614												

Table 1 (continued)

Gene	AGI code	P	E	U	S	FC	Gene	AGI code	P	E	U	S	FC
ENTH domain containing proteins													
ENTH1	At2g4316	*			234		ENTH4	At1g0867					
ENTH2	At3g5929	*	*	*	1937	15.5	ENTH5	At3g4654					
ENTH3	At5g1171	*			400		ENTH6	At3g2335	*	*	*	159	5.0

bears unique sequence motifs. Also other pollen-enriched isoforms, PIPK4-6, form a specific cluster in the phylogeny of type B PIP kinases (Mueller-Roeber and Pical 2002), further indicating pollen-specific regulation of PIP_2 synthesis. As noted previously, despite the absence of PI3-kinase, 3 of 4 Fab1 genes, coding for putative PI3P-5 kinase, are expressed in pollen. PITPs (Sec14p nodulin domain phosphatidylinositol transfer proteins) are characterised by their ability to transfer PI or PC monomers between membrane bilayers in vitro. PITP dependence has been observed in reconstitutions of constitutive exocytosis, regulated exocytosis, intra-Golgi membrane trafficking, and plasma membrane signalling, suggesting that PITPs play important roles in regulating PPI production in vivo (Routt and Bankaitis 2004). Vincent et al. (2005) recently showed that AtSfh1, member of plant PITP family, controls PIP_2 synthesis and regulates cytoskeleton in root hairs, demonstrating the necessity of PITPs for tip growth.

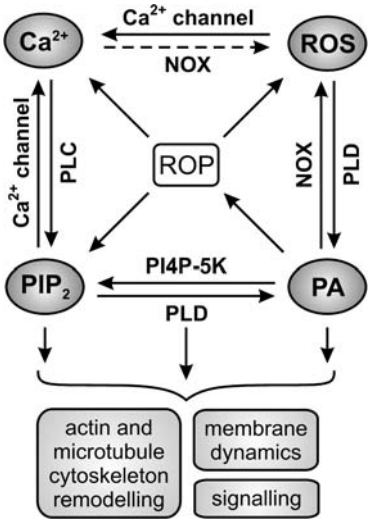

Fig. 3 Hypothetical scheme depicting suggesting central role of PIP_2/PA tandem in regulation of tip growth in pollen tubes

PPI degradation genes exhibit trends similar to the PIP_2 synthesis pathway. From 15 genes coding for inositol polyphosphate 5-phosphatases (5PTases), 4 loci are expressed and upregulated in pollen. Three of these genes, 5PTase12, 5PTase13 and 5PTase14, belong to type II subfamily of 5PTases, which in yeast and animals have been known to regulate vesicle trafficking and actin organization (Suchy and Nussbaum 2002). 5PTase14, the most abundant pollen isoform, has highest substrate specificity to PIP_2, while 5PTase12 and 5PTase13 hydrolyse only Ins(1,4,5)P_3 (Zhong and Ye 2004). The remaining member of plant type II 5PTase family, 5PTase15, has been demonstrated recently to be the FRA3 gene, which controls actin organisation and secondary cell wall synthesis in fiber cells (Zhong et al. 2004), hence analogous functions can be hypothesised for pollen isoforms. Actin disorganisation was also shown in *fra7* mutant, coding for SAC domain containing PPI phosphatase that preferentially cleaves membrane PPIs (Zhong et al. 2005).

Expression data indicate that PIP_2-regulated PLDs, particularly PLDδ and PLDβ1 are involved in pollen PPI signalling (Fig. 2). PLDβ1 interacts with the actin cytoskeleton (Kusner et al. 2003) and PLDδ is connected to ROS-mediated stress signalling in vegetative cells (Zhang et al. 2003) and our data suggest that these isoforms fulfil similar roles in pollen tubes (Potocky et al., unpublished data).

Over the past ten years, several PPI-binding modules have been recognised: pleckstrin homology (PH) domains, which may bind broad spectrum of PPIs such as PI(4,5)P_2, PI(3,5)P_2, PI(3,4)P_2, PI3P, PI4P, PI5P and other acidic phospholipids such as PA with various specificity and affinity (Yu et al. 2004), FYVE domains, which are specific towards PI3P, PX domains, which are mostly specific to PI3P, and ENTH domains, which are mostly specific for PI(4,5)P_2 (Lemmon 2003). From 53 *Arabidopsis* PH domain-containing proteins, 26 are present in pollen transcriptome, covering all 11 subfamilies (van Leeuwen et al. 2004). 12 genes from 7 subfamilies are upregulated in pollen, including regulators of small GTPases (ArfGAP3, RhoGap1, putative RabGEFs Praf2, Praf6, Praf7, also containing FYVE domain), putative oxysterol-binding proteins (OBP2) and proteins bearing the PH domain and other putative lipid-binding module, START domain (START1, START2 and START5). Interestingly, protein kinase PDK1-1, a PH domain containing protein, which is specifically activated upon PA binding (Anthony et al. 2004) is also upregulated in pollen, whereas both its known target AGC2-1 and its interaction partner AGC1-1are absent from pollen. Similarly, 4 out of 6 ENTH domain are called "present in pollen" with 2 pollen selective genes (Table 1). On the other hand, none of the PX domain and FYVE domain proteins is selective or enriched in pollen (with the exception of Praf proteins, which bear also PH domain, see above), providing another indirect clue to PIP_2 as central phosphoinositide in pollen.

4.2 PTEN-like Domains as Possible Targets of Lipid Signalling

Proteins of the PTEN superfamily are emerging as candidates for a link between signalling lipids, in particular $PI(3,4,5)P_3$ (PIP_3), and downstream effectors. The PTEN molecule exhibits structural similarity to dual specificity protein phosphatases, contains the protein phosphatase active site signature, HCXXGXXR, and possesses a phosphatase activity towards both lipids and proteins. However, its protein tyrosine phosphatase activity is rather weak, compared to the strong affinity towards PIP_3 (Li and Sun 1997).

Gupta et al. (2002) identified three PTEN homologues in the *Arabidopsis* genome and demonstrated that one of them, AtPTEN1 (At5g39400), exhibits a lipid- and protein-phosphatase activity similar to the mammalian prototype. The failure to isolate T-DNA insertion mutants in AtPTEN1 suggested that the gene might be essential, which led the authors to construct "knock-down" *Arabidopsis* lines using the RNAi technology. They found that AtPTEN1 depletion results in loss of pollen viability, which is consistent with the observed pollen-specific activity of the AtPTEN1 promoter. The affected pollen grains revealed cell surface defects, namely local separation of plasma membrane from the intine, as well as both intine and exine lesions, again supporting possible role of PTEN in development of surface structures of the pollen grain. Interestingly, the AtPTEN proteins (and their relatives from other species) are not the only representatives of the PTEN superfamily in the plant kingdom. A divergent PTEN-related domain is present in the variable N-terminal extension of many plant formins (Cvrčkova et al. 2004b), i.e., members of the formin (FH2 protein) family.

Higher plants have a prolific family of FH proteins (encoded by 21 loci in *Arabidopsis*). Plant formins can be divided into two distinct subfamilies (classes) based on the sequence of the conserved FH2 domain (Cvrčkova et al. 2004a; Deeks et al. 2002). Presence of PTEN-like domains is characteristic for Class II plant formins, while most Class I proteins carry a N-terminal membrane-insertion signal (Cvrčkova 2000), suggesting a possible plant-specific mechanism of cytoskeleton-membrane connection. No functional studies on Class II formins have been reported so far. It is not even known yet whether Class I and Class II FH2 proteins can form mixed heterodimers, which would greatly increase the diversity of formin complexes and their regulation. In *Arabidopsis* pollen, only three Class II formins (AtFH13, AtFH14 and AtFH17) are expressed to a significant extent (data from Honys and Twell 2004 and Pina et al. 2005); two of them, AtFH13 and AtFH14, possess the PTEN-related domain.

The only available knowledge on the PTEN-like domain present in many Class II formins results from bioinformatic analyses of a collection of the four domains of *Arabidopsis* formins AtFH13, AtFH14, AtFH18 and AtFH20, as well as related sequences from rice and *Medicago truncatula* (Cvrčkova

et al. 2004a). Surprisingly, all these formin-associated PTEN domains contain mutations that make catalytic activity as either lipid or protein phosphatase extremely unlikely. A crucial arginine residue in the phosphatase active site is replaced by hydrophobic or small polar residues in the plant proteins, and a conserved and functionally important asparagine is substituted by glycine. We therefore believe that the function of plant PTEN domains is rather structural than catalytic – perhaps analogous to the role of the transmembrane segments in Class I formins. A variant PTEN domain that lacks catalytic activity but retains its intracellular phospholipids-dependent localization ability might contribute to intracellular positioning of the actin-organizing FH2 domains. Such a structural role could perhaps be attributed to the C-terminal portion of the conserved PTEN core, which is related to a class of domains collectively referred to as C2; curiously, this portion appears to be lost in the AtPTEN proteins, while it has been retained in the formin-associated PTEN domains, albeit in a highly diverged form (Cvrčkova et al. 2004a; Gupta et al. 2002). The C2 domains found in PTEN-like part of plant formins resemble the C2 domain of human PTEN, including the residues that make PTEN Ca^{2+} independent.

5 Perspectives

There are two major aspects of phosphoinositides function in eukaryotic cells – as signalling molecules and as localization cues, enabling the recruitment of phosphoinositide-binding proteins to specific phosphoinositide-containing membranes or membrane domains (Carlton and Cullen 2005). In pollen tubes these two aspects are likely to relate. A dynamic gradient in plasmalemma lipid composition from the growing tip to less active sub-apical, non-growing regions can be expected. The paradigm for secretory pathway and tip growth in plants claims that Golgi-produced secretory vesicles are vectorially transported, along F-actin cables, towards the growing cell walls or tips where they accomplish local exocytosis. Fusing vesicles deliver both new cell wall material and membranes for extending plasma membrane. Endocytosis is implicated in this classical model to remove the excess of the inserted membranes. However, recent data revealed new aspects that urge a revision of this model. In vivo observations of the vesicle-rich apical zone in both root hairs and pollen tubes revealed large amounts of vesicles that move chaotically throughout this clear zone and which only seldom accomplish complete fusion with the apical plasma membrane (Ovecka et al. 2005). Surprisingly, in both pollen tubes and root hairs exposed to endocytic tracers FM1-43 and FM4-64 all secretory vesicles of the clear zone rapidly become labelled; the internalization – i.e., endocytosis – of tracers is very rapid pointing to the overlap between pollen tube exocytosis and endocytosis (Ovecka et al. 2005;

Samaj et al. 2005). Similar data have been obtained with the recycling endosome marker GFP-RabA/Rab11 which accumulated within the clear zone of tip-growing pollen tubes (de Graaf et al. 2005). Endocytosis and endosomes are known to be tightly linked to the actin cytoskeleton and lipid domains enriched with structural sterols play an active role in this process (Golub and Caroni 2005; Yin and Janmey 2003). Ectopic expression of activated AtRAC10 disrupted the actin cytoskeleton, compromised endocytosis, and resulted in aberrant root hair formation (Bloch et al. 2005). Interestingly, AtRAC10 localizes to an insoluble fraction of the plasma membrane suggesting that it is enriched within lipid rafts of plant cells. Our unpublished data revealed accumulation of filipin-positive sterols within outgrowing bulges and at tips of growing but not non-growing root hairs (Ovecka et al., in preparation); in pollen tube, however, filipin did not result in differential staining of the apex. Recent analyses of pollen GPI proteins showed that lesions in some of them results in reduced pollen germination and tube growth (Lalanne et al. 2004). As GPI anchored proteins are well known "inhabitants" of cholesterol enriched lipid rafts their prominent representation in pollen transcriptome suggests a role of lipid rafts in pollen tube growth regulation.

PIP_2 is highly enriched at the tips of root hairs (Braun et al. 1999) and AtSfh1p is essential for the enrichment of PIP_2 at tips of root hairs as well as for the maintenance of clear zone vesicles and dense meshworks of F-actin (Bohme et al. 2004; Vincent et al. 2005). AtSfh1p might stimulate activity of PLD that would result in PIP_2 synthesis on the clear zone vesicles via activation of PIPK by PA. This suggests a presence of reciprocal positive feedback regulation of PA and PIP_2 enabling creation of distinct PA and PIP_2 enriched membrane domain at the expanding pollen tube tip or root hair (Fig. 3). Kusner et al. (2002) has shown that F-actin not only binds but also activates plant PLD, while G-actin inhibits its activity. Activation of PLD thus results in PA generation, which might feed into another lipid-signalling pathway – PA-induced and phosphoinositide-dependent kinase-1 (PDK1) stimulation (Anthony et al. 2004). PDK1 is expressed in pollen but further data are required to assign a role to this signalling pathway.

Another signalling pathway that might crosstalk with phosphoinositides is the Reactive Oxygen Species (ROS) pathway. We have recently described polarized and calcium stimulated NADPH-oxidase (gp91 NOX subunit homologue)-dependent ROS production at the tips of growing pollen tubes (Jones et al. submitted), similar to the ROS production machinery of root hair tips (Foreman et al. 2003). In the case of pollen tubes we hypothesize that, just like $[Ca^{2+}]_c$, ROS production oscillates with the growth rate oscillation (Jones et al. submitted). From this point of view we also reinterpret the negative chemotaxis of pollen tubes towards nitric oxide (NO; Prado et al. 2004) as an outcome of inhibitory or interference effects of NO on NOX activity or ROS metabolism at the tip (Fujii et al. 1997). We predict that significant proportion of O_2 consumption accompanying pollen tube germination and

growth is brought about by NOX catalyzed ROS production and NO production. NADPH oxidase producing ROS molecules may also be localized to endosomes. Rho/Rac GTP-binding proteins are well known activators of NOX dependent ROS production also in plants (Kawasaki et al. 1999; Moeder et al. 2005; Carol et al. 2005) and recently described oscillations in Rop activity at the pollen tube tip (Hwang et al. 2005) might account, along with Ca^{2+}, for putative NOX dependent ROS oscillations in a feed-back regulatory circuit comprising activation effect of Ca^{2+} and regulatory effects of activated Rop on NOX activity. PA in plant cells can induce ROS production (Zhang et al. 2005), acting probably via Rops (Park et al. 2004). Recently discovered family of plant specific Rop GEFs (Berken et al. 2005) and their interaction with pollen receptor kinases (Kothien et al. 2005) allows us to hypothesize, that PA might activate Rops by changing lipid environment of receptor kinases. We suggest the possibility that Rop, Ca^{2+}, putative NOX/ROS oscillations and PA/PIP_2 reciprocal regulation are components of a positive feed-back process producing a distinct tip-localized membrane domain facilitating highly polarized cell expansion of pollen tubes (Fig. 3).

Considering the accumulation pattern of recycling proteins and pectins, tips of pollen tubes/root hairs and cytokinetic cell plates resemble endocytic BFA-induced compartments (Dhonukshe et al. 2006; Samaj et al. 2004), suggesting that similar endocytic secretory pathways drive tip growth and cell plate formation. The majority of vesicle trafficking at the pollen tube tip is most probably related to the activity of pollen specific recycling endosome, an assumption supported by our recent discovery of known Rho and Rab effector exocyst complex and their importance for pollen tube growth (Elias et al. 2003, Zarsky et al. 2004, Cole et al. 2005).

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The Actin Cytoskeleton in Pollen Tubes; Actin and Actin Binding Proteins

Etsuo Yokota (✉) · Teruo Shimmen

Department of Life Science, Graduate School of Life Science, University of Hyogo, Harima Science Park City, 678-1297 Hyogo, Japan
yokota@sci.u-hyogo.ac.jp

Abstract Actin cytoskeleton is well known to be a key element for the germination and the elongation of pollen tubes. It has been appreciated that the cytoplasmic streaming for conveying secretory vesicles to the tube apex is a primary function of the actin cytoskeleton in pollen tubes. Recently growing evidence has revealed that highly dynamic populations of actin cytoskeleton are present in apical and subapical regions of tubes and are involved intimately in polar elongation of tubes. Tip-focused Ca^{2+}-gradient and tip-localized small GTPases (Rop/Rac) are believed to control such actin dynamics through the various kinds of actin binding proteins (ABPs). In the present chapter, we focus on the organization of actin in elongating pollen tubes and characterization of ABPs identified from pollen. We further discuss their roles, with special emphasis on recently identified proteins of the gelsolin family, regulating actin dynamics and organizing actin architecture in pollen tubes.

1 Introduction

Actin is one of the most ubiquitous and highly conserved proteins among eukaryotic cells. Under physiological ionic conditions, monomeric actin (G-actin) is polymerized into filamentous polymers (F-actin or actin filament) through the nucleation step for forming intermediates, such as dimers and trimers. F-actin is a polar filament that adds preferentially G-actin on its barbed (plus) end, and tends to release G-actin from opposite pointed (minus) end. Many kinds of ABPs are responsible for regulating and modulating actin turnover or dynamic balance between polymerization and depolymerization and for organizing actin filaments into highly ordered structures. Myosin is an actin-based molecular motor moving and sliding along actin filaments. These three components form the actin cytoskeleton, which is involved in various cellular functions and activities in eukaryotes.

2 Actin

In higher plants, actin genes constitute a multi-gene family and are subdivided into two classes, which differ in their expression pattern, reproductive and vegetative. *Arabidopsis* has at least eight expressed actin genes, *ACT1*, *ACT2*, *ACT3*, *ACT4*, *ACT7*, *ACT8*, *ACT11*, *ACT12* (Meagher et al. 2000). *ACT2*, *ACT7* and *ACT8* are strongly expressed in vegetative tissues, while *ACT1*, *ACT3*, *ACT4*, *ACT11* and *ACT12* in reproductive tissue and pollen. The cytosolic concentrations of actin in pollen were estimated to be around 100 μM and 250 μM, corresponding to about 5% and 2.3% of total extractable protein in maize (Gibbon et al. 1999) and poppy (Snowman et al. 2002), respectively, and did not change significantly during the germination. In lily pollen, however, concentration before germination was estimated to be ~ 25 μM and increased about 70% after 2 hr of germination (Vidali and Hepler 1997). In either case, actin is an abundant protein in pollen. The concentration of F-actin, however, was estimated to be in the order of tens of μM in maize (Gibbon et al. 1999) and poppy pollen (Snowman et al. 2002), which is significantly lower than that of G-actin. This indicates that large part of the actin protein is in the monomeric form.

Functionally active actin was isolated from maize pollen by two independent methods, acetone-treatment (Liu and Yen 1992, 1995), commonly used in preparing the actin from muscle tissues, and a poly-L-proline (PLP) affinity column using human recombinant profilin (Ren et al. 1997). In the later case, G-actin in a crude protein extract from pollen binds to exogenously added human profilin and then is adsorbed on the PLP column via an association between profilin and PLP (see section below). Subsequently, G-actin is selectively eluted with high ionic strength solution from the column while profilin remains bound. This method is generally used to prepare pollen actin, because of rapid, reproducible and convenient procedure. The biochemical characterization revealed that pollen actin binds to subfragments of muscle myosin, shows kinetics of polymerization similar to muscle actin, and can be incorporated into the array of actin filaments in living plant cells, such as stamen hair cells of *Tradescantia*. Hence, pollen actin has similar properties to non-plant actin. Interestingly, pollen actin was predominantly incorporated into pollen F-actin in vitro and into the array of actin filaments in stamen hair cells (Ren et al. 1997; Jing et al. 2003). In contrast, animal G-actin preferentially co-polymerized with animal G-actin and F-actin. These results indicate the functional diversity between animal and pollen actin, despite high conservation (up to 80%) in their primary structures.

3
Organization of Actin Cytoskeleton in Pollen and Pollen Tubes

During the germination of pollen, the organization and architecture of actin filaments are dramatically altered (Haslop-Harrison and Heslop-Harrison 1992; Smertenko et al. 2001). Before hydration, actin seems to be tightly packed in fusiform or spicule structures and aggregates, presumably storage forms of actin. During the hydration, actin filaments dissociate from these structures, and then are organized into fine bundles dispersed randomly in grains. A network of actin filaments concentrates on the germinal aperture(s) before the germination, and eventually actin-filament bundles are ramified in the pollen tubes.

In elongating pollen tubes, there appears to be three major regions of distinct actin-filament arrangement and architecture: apical, subapical and shank region (Vidali and Hepler 2001). In the shank of tubes, actin filaments are organized into bundles arranged in a longitudinal or a helical orientation throughout the cytoplasm. These actin filaments, together with myosin, generate the cytoplasmic streaming for transport of organelles and Golgi-driven secretory vesicles containing cell wall materials (Geitmann and Steer, this volume), which are fused at the tip and participate in the elongation of tubes. The cytoplasmic streaming in pollen tubes frequently exhibits a reverse fountain pattern with the streaming occurring acropetally along the edge or side of tubes, and basipetally in central or core region (Vidali and Hepler 2001).

The actin organization in apical and subapical regions is still unclear with contradictory reports, most likely a result of different methodologies and species under study (Vidali and Hepler 2001). The expression of the F-actin binding domain of talin tagged with GFP (Kost et al. 1998), provided the first live images of the pollen tube actin cytoskeleton and made it possible to trace and analyze the dynamics in close to real time. However, tube growth after GFP-talin expression is strongly perturbed raising questions about the validity of this methodology. In spite of the ongoing debate, it is accepted that a few short actin filaments or fine and thin bundles are dispersed with random orientation in the apical regions, while a ring-like arrangement of actin filaments is found in the subapical regions (Fig. 1). This ring-like arrangement has been coined as collar (Kost et al. 1998), subapical mesh (Chen et al. 2002; Geitmann et al. 2000) basket (Snowman et al. 2002), funnel (Vidali et al. 2001) or fringe (Lovy-Wheeler et al. 2005). Recently, this arrangement has been carefully evaluated with state-of-the-art fixation regimes (Lovy-Wheeler et al. 2005) and has been found to consist of longitudinal actin filaments concentrated densely in the tube cortex. The apical regions are devoid of large organelles and exhibit a chaotic-like cytoplasmic streaming. Therefore, this area occupied by numerous secretory vesicles is also called clear zone. The actin cytoskeleton in the apical region seems to relate with the suppression of cytoplasmic streaming in this region with thinner and transient microfil-

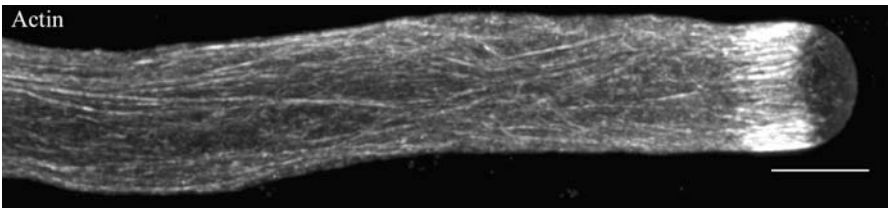

Fig. 1 Pollen tubes of lily exhibit a marked cortical fringe of actin in the apical domain. In the shank, back from the tip, actin microfilaments are evenly dispersed throughout the thickness of the tube. The pollen tube was prepared first by rapid freeze fixation, and freeze substitution. It was then rehydrated, and labeled with anti-actin antibody. (from Lovy-Wheeler et al. 2005, reprinted with permission)

aments occuring next to the plasma membrane within 5 μm from the tip (Fu et al. 2001). The absence of microfilaments at the tip of the growing tube was suggested to be due to the tip-focused Ca^{2+} gradient indispensable for growth (Hepler et al., this volume).

4
Characterization of ABPs Identified in Pollen

ABPs bind to and interact with both G-actin and F-actin in various manners (e.g., side-binding, end-capping, and cross-linking of filaments). In pollen tubes, ABPs are intimately involved in the arrangement of actin filaments into bundles and the regulation of actin dynamics or turnover, polymerization or depolymerization. Thus far, three types of ABPs, have been identified from pollen: profilin, ADF (actin depolymerizing factor)/cofilin and gelsolin family proteins – fragmin, gelsolin and villin. Their biochemical properties and localization in pollen or pollen tubes have been extensively studied (Drøbak et al. 2004; Staiger and Hussey 2004); profilin and ADF/cofilin regulate actin filament assembly and act as increasing the turnover of filaments while the gelsolin family proteins function in both regulating actin dynamics and organizing architectures of actin filaments.

4.1
Profilin

Profilin is small G-actin-binding protein (12 to 15 kDa) that forms a 1 : 1 complex with G-actin, and also binds to phosphoinositides, proline-rich proteins and PLP (Drøbak et al. 2004; Staiger and Hussey 2004). This protein was originally identified as an allergen of birch pollen (Valenta et al. 1991). Plant profilin comprises a multigene family containing 5 to 10 isoforms in many plant tissues, and the pollen is no exception. *Arabidopsis* has five isoforms of profilin,

PRF1 to PRE5 (Kandasamy et al. 2002). Among them, PRF4 and PRF5 are specifically expressed in mature pollen and pollen tubes, but not found in other cell types (including microspores), while PRF1, PRF2 and PRF3 are constitutive in all vegetative tissue at various stages of development. These isoforms are also expressed predominantly in ovules and microspores at the early stages of miscrosporogenesis. In maize, five isoforms, ZmPRO1 to ZmPRO5, are expressed in pollen (Kovar et al. 2000a). ZmPRO1 is abundant in pollen, while ZmPRO4 and ZmPRO5 are predominantly expressed in vegetative cells or tissues and insufficiently in pollen. In pollen tubes, profilin is uniformly distributed throughout the cytoplasm in lily (Vidali and Hepler 1997), *Arabidopsis* and tobacco (Kandasamy et al. 2002). In tobacco pollen tubes, profilin is also localized in the vegetative nuclei, but not in sperm cells. Pollen contains profilin abundantly, and its concentration (Gibbon et al. 1999; Vidali and Hepler 1997; Snowman et al. 2002) was nearly the same as that of total actin protein, indicating that most of profilin likely forms complexes with actin monomer.

Plant profilin shares functional and structural similarity with animal profilin (Drøbak et al. 2004; Staiger and Hussey 2004), although it does not accelerate the nucleotide exchange rate of G-actin (Perelroizen et al. 1996). This protein has two opposite actions on the dynamics of actin in vitro. Profilin depolymerizes actin filaments by binding to G-actin, and then sequesters G-actin from the spontaneous nucleation of polymerization. Conversely, the profilin-actin complex promotes and enhances the polymerization of G-actin from uncapped and free barbed ends of actin filaments when a large pool of G-actin is present. Maize profilins appeared to be divided into two functional classes possessing different affinities for G-actin and PLP (Kovar et al. 2000a). Class II profilin, ZmPRO4 and ZmPRO5, showed higher affinity for PLP and stronger sequestering activity of G-actin than those of class I profilin, ZmPRO1, ZmPRO2 and ZmPRO3. Hence, class II profilin microinjected into stamen hair cells, induced the disruption of cytoplasmic architecture and then displacement of nucleus via the depolymerization of actin filaments more efficiently and rapidly than class I profilin. Furthermore, the ability of both classes of maize profilins to sequester G-actin was enhanced by Ca^{2+}. This property is believed to contribute to the regulation of actin dynamics in apical and subapical regions of pollen tubes as described below. Interestingly, it was reported that poppy pollen profilin alters the phosphorylation level of several pollen proteins (Clarke et al. 1998). This evidence raised a possibility that pollen profilin is involved in signaling pathway by regulating protein kinase and phosphatase activities.

4.2 ADF/cofilin

ADF/cofilin is another small ABP (15 to 19 kDa) which enhances and modulates actin filament dynamics (Maciver and Hussey 2002; Drøbak et al. 2004;

Staiger and Hussey 2004). It increases actin-filament ends available for polymerization by severing filaments and dissociates actin monomers from the pointed ends of filaments for assembly at the barbed ends. In *Arabidopsis*, at least nine members of ADF/cofilin proteins (AtADF1 to AtADF9) are known to be present (Dong et al. 2001). Some isoforms are expressed predominantly in vegetative tissues, others primarily in reproductive organs. In maize, three genes encoding this protein, ZmADF1, ZmADF2 and ZmADF3, have been characterized, and are differently expressed in maize plant (Lopez et al. 1996). ZmADF1 and ZmADF2 are expressed exclusively in pollen and ZmADF3 in other tissues. The localization of ADF/cofilin in pollen was shown to be altered during maturation and germination (Smertenko et al. 2001), similarly to actin. In mature pollen grains, the protein co-localizes with actin filament arrays whereas in dehydrated pollen grains, some was co-localized with the storage form of actin (e.g., spicules or aggregates). During the germination, ADF/cofilin was accumulated at the sites of pollen tube emergence, and this pattern seemed to depend on the actin network. In pollen tubes, ADF/cofilin distributed evenly in the cytoplasm but interestingly, at points of adhesion of the pollen tube tip to an adjacent substrate, the protein appeared to bind to actin filaments (Smertenko et al. 2001). In contrast to these findings, when tobacco pollen-specific ADF/cofilin (NtADF1) tagged with GFP was expressed at moderate level in tobacco pollen, it bound and decorated predominantly subcortical mesh of actin filaments and their bundles in the shank of pollen tubes (Chen et al. 2002).

The actin-severing and -depolymerization activities of plant ADF/cofilin are sensitive to pH, and further regulated by phosphorylation and phosphoinositide 4,5 bisphosphate (PtdIns(4,5)P_2) (Maciver and Hussey 2002; Drøbak et al. 2004; Staiger and Hussey 2004). At low pH, this protein binds preferentially and cooperatively to actin filaments, while at higher pH, it promotes the rapid depolymerization of filaments by severing and accelerating mononer dissociation from the pointed ends of filaments. The depolymerizing activity of pollen-specific ADF/cofilin, lily LIADF1 (Allwood et al. 2002) and tobacco NtADF1 (Chen et al. 2002) was also enhanced at higher pH. The Ser 6 in vegetative type maize ADF/cofilin, ZmADF3 molecule, was phosphorylated by the calmodulin (CaM)-like domain protein kinase (CDPK), and as a result, its actin-binding and depolymerizing activities were reduced (Smertenko et al. 1998; Allwood et al. 2001). Similar reduction of activities of ZmADF3 (Smertenko et al. 1998) and AtADF1 (Ressad et al. 1998), a vegetative type of *Arabidopsis* ADF/cofilin protein, was induced when Ser 6 of these ADF/cofilin was replaced with Asp to mimic a phosphorylation state. In the elongating pollen tubes, a high activity of Ca^{2+}-dependent protein kinase was reported in the apex (Moutinho et al. 1998). These data suggests that the activity of ADF/cofilin in pollen tubes is also controlled by CDPK through phosphorylation and dephosphorylation. However, lily pollen LIADF1 was not found to be phoshorylated by CDPK (Allwood et al. 2002). In tobacco pollen NtADF1 re-

placement of Ser 6 by Asp led to a significant reduction in the interaction with actin filaments (in vitro and in vivo) (Chen et al. 2002). Both phosphorylated and unphosphorylated ADF/cofilins were present at similar concentrations in mature pollen of tobacco (Chen et al. 2003). These results suggested that the phosphorylation at Ser6 of NtADF1, which is not catalyzed by CDPK, is an important regulatory mechanism for its activity in pollen tubes. Furthermore, the Rop/Rac signaling pathway, (Hwang and Yang, this volume) was suggested to be involved in the phosphorylation of ADF/cofilin. Overexpression of tobacco pollen Rac1, NtRac1 induced the isotropic growth resulting in pollen tubes with ballooned tips and a disrupted actin cytoskeleton; these phenomena were diminished by the co-expression of tobacco pollen NtADF (Chen et al. 2003). When Ser 6 was replaced with Asp, the activity of NtADF1 to counteract the effect of NtRac1 decreased. Moreover, the binding activity of expressed NtADF1 to actin filament arrays in pollen tubes was suppressed by co-expression of NtRac1 or constitutively active form of NtRac1 while the phosphorylation level of NtADF1 increased. These results further support that the phosphorylation of Ser 6 in ADF/cofilin plays a critical role in controlling its activity, and suggested that ADF/cofilin is one of down regulators in signaling pathway of Rop/Rac in pollen tubes (Chen et al. 2003).

The binding activity of LIADF1 to f-actin was also inhibited by specific phosphoinositides, such as PtdIns(4,5)P_2 (Allwood et al. 2002), probably by direct binding to the actin-binding site(s). In addition to these regulatory mechanisms described above, the depolymerizing activity of LIADF1 was found to be dramatically enhanced in the presence of another pollen protein called actin-interacting protein 1 (AIP1; Allwood et al. 2002). There are two AIP1 genes, *AtAIP1-1* and *AtAIP1-2*, in *Arabidopsis*. AtAIP1-1 is expressed in floral tissues but not in vegetative organs, whereas AtAIP1-2 is in all tissues containing floral tissues. In daffodil pollen, AIP1 protein showed a distribution patter similar that of to ADF/cofilin during the maturation, hydration, and germination of pollen (Smertenko et al. 2001). These observations raise the possibility that AIP1 regulates and/or modulates the activity of ADF/cofilin in pollen tubes.

4.3 Gelsolin Family Protein

Gelsolin family proteins are generally composed of several gelsolin (or gelsolin/severin) repeat domains; fragmin and gelsolin have three and six of those domains, respectively, while villin has a headpiece domain in addition to the six gelsolin repeat domains (Drøbak et al. 2004; Staiger and Hussey 2004). Gelsolin family proteins show various functions in regulating actin dynamics and organizing actin filaments in a Ca^{2+}-dependent manner. In the presence of Ca^{2+}, these proteins bind to G-actin and form the complex which works as a nucleator for polymerization of actin, thus resulting in the

acceleration of polymerization (referred to as nucleating activity). Furthermore, they sever actin filaments and remain attached the barbed ends of severed filaments as a cap. As a result, short actin filaments that can not anneal with each other and not elongate at their barbed ends, are produced. PtdIns(4,5)P_2 dissociates gelsolin family proteins from G-actin and barbed ends of filaments. In addition, villin arranges actin filaments into bundles at low concentrations of Ca^{2+} through its headpiece domain. Plant homologues of villin (P-135-ABP and P-115-ABP; Vidali et al. 1999; Yokota et al. 2003), gelsolin (PrABP80; Huang et al. 2004) and fragmin (LdABP41; Fan et al. 2004) were identified from the pollen of *Lilium longiflorum*, *Papaver rhoeas* and *Lilium davidii*, respectively.

80-kDa PrABP80 and 41-kDa LdABP41 were originally isolated from pollen by using a DNase I affinity column chromatography (DNase I possesses strong binding activity to G-actin, in addition to DNA degradation activity) (Huang et al. 2004; Fan et al. 2004). In the presence of Ca^{2+}, both gelsolin family proteins bound to endogenous G-actin in the crude pollen protein extract, and adsorbed on the column via the interaction of G-actin with DNase I. In a final separation step, PrABP80 and LdABP41 were eluted from the column with EGTA, while G-actin remained bound to the column. Lily villin in the crude protein extract exhibited a behavior on this column similar to that of PrABP80 and LdABP41 (Yokota et al. 2005). The biochemical properties of PrABP80 have been extensively studied, though its localization in pollen has not been shown. PrABP80 could act as a nucleus for actin polymerization, resulting in the acceleration of polymerization, higher rate of barbed end capping and Ca^{2+}-dependent severing (Huang et al. 2004). Furthermore, this protein exhibited preferential binding to certain phosphoinositides, such as PtdIns(3,5)P_2, PtdIns(3,4)P_2 and PtdIns(5)P, and phosphatidic acid, but relatively less binding to PtdIns(4,5)P_2 and PtdIns(3,4,5)P3 (Drøbak et al. 2004). The influence of these phosphoinositides and phosphatidic acid on the activity of PrABP80 has however not been studied.

Fragmin-like protein, LdABP41, also exerted G-actin-nucleating and actin-filament-depolymerizing activities in the presence of Ca^{2+} (Fan et al. 2004). This protein was found to be concentrated preferentially in the apical and subapical regions of elongating pollen tubes (Fan et al. 2004) and microinjection of the antibody against LdABP41 into the germinating pollen induced the suppression of pollen tube elongation. In the *Arabidopsis* genome database, no genes for gelsolin and fragmin have been found (Huang et al. 2004; Staiger and Hussey 2004; Fan et al. 2004). Based on the crossreactivity with antibody against villin and an analysis of partial amino acid sequences, PrABP80 and LdABP41 were suggested to be expressed by alternative splicing from villin gene(s).

Plant villins, P-135-ABP and P-115-ABP, have been originally identified as actin-filament bundling factors from the germinating pollen of lily (Yokota et al. 1998; Nakayasu et al. 1998). They arrange actin filaments into bundles

with same polarity (Yokota and Shimmen 1999; Yokota et al. 2003), and this activity was suppressed in the presence of both Ca^{2+} and calmodulin (CaM), but not by Ca^{2+} alone (Yokota et al. 2000, 2003). P-135-ABP co-localized well with actin-filament bundles in the shank region of lily pollen tubes (Yokota et al. 1998; Vidali et al. 1999). P-135-ABP and P-115-ABP contained in the pollen crude protein extract from lily also associated with a DNase I column in the presence of Ca^{2+} and eluted with EGTA, indicating that both ABPs bind to G-actin in a Ca^{2+}-dependent manner (Yokota et al. 2005). Furthermore, P-135-ABP showed nucleating, end capping and depolymerizing activities, although the severing activity has not been found. However, the nucleation and depolymerization efficiencies of P-135-ABP appeared to be lower than those of PrABP80. In *Arabidopsis*, five villin isoforms are known to be present (AtVLN1∼AtVLN5), and AtVLN2 and AtVLN5 are mainly expressed in pollen (Huang et al. 2005). In VILLIN1 (AtVLN1) the amino acid sequences deduced to provide Ca^{2+}-sensitivity are poorly conserved (when compared to other *Arabidopsis* isoforms), and the recombinant protein showed only F-actin binding and bundling activities in a Ca^{2+}- and Ca^{2+}-CaM-insensitive fashion (Huang et al. 2005). The F-actin bundles formed by AtVLN 1 were stable and resistant to the depolymerizing action of *Arabidopsis* ADF/cofilin, AtADF1.

5
Actin Dynamics in Pollen Tubes

Several recent studies have revealed that pollen tube growth depends not only on the presence of stable actin bundles for cytoplasmic streaming, but also on the dynamic state of actin in the tip region. Actin depolymerizing drugs, latrunculin B and cytochalasin D (Gibbon et al. 1999; Vidali et al. 2001), and G-actin binding proteins, profilin and DNase I (Vidali et al. 2001) microinjected into pollen, inhibited pollen tube growth at concentrations lower than those needed to suppress the cytoplasmic streaming. Furthermore, an aberrant actin array was frequently observed in tip regions of tubes treated with these reagents. Despite the potential problems of the technique, images of the actin cytoskeleton in live tobacco pollen tubes expressing talin-GFP, showed that the short and fine actin-filament bundles in the apical and sub-apical regions are a highly dynamic structure (Fu et al. 2001). The emergence of short actin bundles was generally associated with reduction or disappearance of actin collar and vice versa. Moreover, the appearance of these structures appeared to oscillate alternately. In elongating pollen tubes, a tip-focused Ca^{2+} gradient (Hepler et al., this volume) and Rops (Hwang and Yang, this volume), are well known to be prominent factor for regulating actin dynamics and organization in tip regions, and so to be indispensable for the tube growth. When the Ca^{2+} gradient was diminished by caffeine, actin-filament

bundles extended and invaded the apical regions of pollen tubes (Miller et al. 1996). In contrast, actin filaments in the pollen tubes were fragmented and depolymerized throughout cytoplasm when cytosolic Ca^{2+} concentrations were elevated in the self-incompatibility response of poppy pollen (de Graaf et al., this volume) or by a Ca^{2+} ionophore, A23187 (Kohno and Shimmen 1987; Snowman et al. 2002). Active Rops localized in the apical region also have a role in regulating the organization and the dynamics of tip localized F-actin (Hwang and Yang, this volume).

Actin dynamics in apical and subapical regions of tubes was shown to oscillate in time and was suggested to be in opposite phase with tube elongation and Ca^{2+} changes (Fu et al. 2001); the peak of polymerization appeared to precede peaks of elongation and elevation of Ca^{2+} concentrations. Recently, it was proposed that Rop1 controls two counteracting pathways regulating the actin dynamics in these regions; one promotes assembly of actin filaments, while a second promotes disassembly by Ca^{2+}-dependent processes (Gu et al. 2005; Hwang and Yang, this volume). If these hypothesis hold true, it seems plausible to postulate the following mechanism for depolymerization of actin filaments: (1) the G-actin binding and sequestering-activities of profilin are enhanced; (2) gelsolin family proteins sever and depolymerize actin filaments, accompanying by capping of the barbed ends of filaments; (3) the polymerization of G-actin from the barbed ends induced by profilin-actin complex is significantly blocked. Furthermore, it is possible that ADF/cofilin also promotes the release of G-actin from the pointed ends of filaments, and profilin sequesters further G-actin, if ADF/cofilin is not phosphorylated in the Rop/Rac signaling network. The depolymerizing activity of ADF/cofilin could increase in subapical regions, due to a constitutive alkaline band (Feijó et al. 1999). However, the existence of such band is still disputed (Messerli et al. 1999). To exert a Ca^{2+}-induced depolymerization by a villin, such as P-135-ABP, micromolar CaM should be needed in addition of Ca^{2+}. CaM was distributed throughout the cytoplasm but it was also reported that the protein appears to bind significantly the sub-apical region in a sort of V-shaped collar (Moutinho et al. 1998). The concentration of CaM in the germinating pollen tubes of lily was estimated to be about 15 μM (Yokota et al. 2004), enough for the depolymerization activity of P-135-ABP. However, the depolymerizing efficiency appeared to be lower than that of gelsolin, suggesting that gelsolin or fragmin predominantly play a main role in fragmentation and depolymerization of actin filaments. Significant actin-filament fragmentation and depolymerization induced by the elevation of cytosolic Ca^{2+} concentrations upon self-incompatible response in poppy pollen is likely to be induced by a mechanism similar to the one described above.

In contrast, the mechanism for promotion of polymerization and elongation of actin at the pollen tube apex is obscure. A key point to consider is that almost all of G-actin should be complexed with profilin, and consequently spontaneous polymerization is tightly suppressed and eliminated. The nucle-

ation activity of gelsolin family proteins, e.g., P-135-ABP (Yokota et al. 2005), can not work when G-actin is saturated and bound to profilin. Two mechanisms for polymerizing and elongating actin filaments are speculated. First, G-actin bound profilin is added to uncapped barbed ends of pre-existing actin filaments (Vidali and Hepler 2001). Second, actin filaments are newly polymerized and created by potential nucleation factor, such as actin related protein Arp2/3 complex or formin. An inactivation of ADF/cofilin by Rop/Rac mediated by phosphorylation is also conceivable to promote actin polymerization.

As to the first mechanism, gelsolin family proteins (or heterodimeric capping proteins) should be released, promoting G-actin addition to free barbed ends. Decreasing Ca^{2+} concentrations is ineffective in dissociating these proteins from actin because their capping activity was almost Ca^{2+}-insensitive. Hence, an additional factor(s), such as PtdIns(4,5)P_2, may be involved in releasing these proteins from barbed ends of filaments. It has been shown that phosphatidylinositol monophosphate kinase that synthesizes PtdIns(4,5)P_2 acts as an effector of tip-localized Rac in pollen tubes (Kost et al. 1999). Hence, dissociating and releasing capping proteins from barbed ends of actin filaments by PtdIns(4,5)P_2 near or at these areas will trigger the elongation of actin filaments. Indirect confirmation of such hypothesis was obtained by localized caged photolysis of PtdIns(4,5)P_2 (Monteiro et al. 2005) which led to a reinforcement of the actin mesh in the sub-apical region of pollen tubes. However, PrABP80 showed relatively low binding ability to PtdIns(4,5)P_2 (Drøbak et al. 2004). Further studies are thus needed to elucidate the mechanism regulating the interaction of gelsolin family proteins with actin.

For the second hypothetical mechanism, two factors, Arp2/3 complex and formin protein, are considered to be good candidates to promote and regulate the polymerization of actin filaments. Arp2/3 complex contains two actin-related proteins, Arp2 and Arp3, and five additional subunits, and the *Arabidopsis* genome contains putative homologues of all seven subunits (Mathur 2005; Szymanski 2005). This complex preferentially binds to the side of pre-existing actin filaments and nucleates daughter actin filaments. This nucleation activity of Arp2/3 complex is enhanced and activated by the WASP (Wiscott-Aldrich syndrome protein)/WAVE (WASP family Verprolin homologous protein)/SCAR (Suppressor of cAR: *Dictyosrelium* homologue of WAVE) family member and recent works identified several genes in *Arabidopsis* encoding plant homologues of some components or subunits of SCAR/WAVE (reviewed in Szymanski 2005). Proline rich domain in these activators is considered to interact with profilin. Moreover, plant homologues, AtSCAR and ZmSCAR distantly related to SCAR/WAVE protein were also identified from *Arabidopsis* and maize, respectively, and found to interact with some components of plant homologue WAVE complex. The components of WAVE complex and Arp2/3 complex were shown to be expressed in floral tissues of *Arabidopsis* but, except for Arp2 (Klahre and Chua 1999), it has not been confirmed whether these proteins are expressed in pollen.

The formin molecule consists of a formin 1 domain (FH1), responsible for profilin binding, and a formin 2 domain (FH2) responsible for actin nucleation (Deeks et al. 2002). *Arabidopsis* has 21 isoforms of formin homology protein (AFH) divided into 2 groups. Group I formin (AFH1 to AFH11) is characterized by N-terminal proline rich glycosylated extracellular domain and a transmembrane domain that targets the formin to the cell membrane (Cheung and Wu 2004; Favery et al. 2004; Ingouff et al. 2005). Overexpression of AFH1 in pollen tubes of tobacco induced the formation of abundant actin cables projecting from the cell membrane, and concomitantly induced pollen tube swelling, growth depolarization and growth arrest (Cheung and Wu 2004). Moreover, the deformation of cell membrane in the apical regions occurred in overexpressing pollen tubes. AFH1 was shown to interact with profilin (Banno and Chua 2000) and recently, the analysis of actin interaction with recombinant constructs containing FH2 alone or FH1 and FH2 domains of AFH1 showed that AFH1 has abilities to promote the polymerization of G-actin bound to profilin (Michelot et al. 2005). The same work revealed that AFH1 interacts with barbed ends of actin filaments forming a "leaky capper" that allows actin polymerization from barbed ends, and binds to the side of filaments for bundling them. AFH5 (AtFH5) has also nucleation and capping activities (Ingouff et al. 2005). AFH2 was expressed in vegetative and floral organs (Banno and Chua 2000), suggesting that AFHs are present in pollen. However, predicted small GTPase binding domain from non-plant formin lacks AFHs. Further studies are needed to elucidate whether the activity of AFHs is controlled by the Rop/Rac signaling pathway and whether AFHs are indeed expressed in pollen tubes.

It remains to be determined how the actin dynamics in tip regions relates to the elongation of pollen tubes. It is possible that dynamic actin assembly provides oscillatory polar guidance to secretory vesicles and determines the position of their fusion at apical cell membrane. A recent work suggested that Ca^{2+} channels at the plasma membrane in tip regions, which are involved indispensably in influx of Ca^{2+} for generating tip-focused Ca^{2+} gradient, are controlled by actin filaments (Wang et al. 2004), and this could generate a positive feed-back for tip growth.

6
The Arrangement of Actin Filaments into Bundles

In the shank regions of pollen tubes where $[Ca^{2+}]_c$ is lower, large parts of the actin filaments are organized into bundles driving the "reverse-fountain" streaming. The direction of movement is dictated by the polarity of actin filaments so they are expected to show opposite directions of polymerization (pointed to barbed ends) at the center and edge of the tubes (Vidali and Hepler 2001). How this is achieved is presently unknown because if it was solely

based on the action of villin (e.g., P-135-ABP and P-115-ABP), the polymerization should be uniform.

In the sub-apical region, the collar structure could be maintained by a Ca^{2+}-insensitive type of villin, such as AtVIL1, which could stabilize the actin bundles and incorporate newly created actin filaments into pre-exist bundles. Fimbrin, another type of F-actin-crosslinking protein, was also identified from plants (McCurdy and Kim 1998), although the presence of this protein in pollen has not been reported. Fimbrin arranged actin filaments into aggregates, instead of bundles, in a Ca^{2+}-insensitive manner (Kover et al. 2000b). If it is expressed in pollen, it is possible that fimbrin is involved in collar formation in subapical regions of tubes.

7
Myosin, the Actin-based Molecular Motor for Cytoplasmic Streaming

Myosin generates the motive force along actin filaments (Shimmen and Yokota 2004) and is the main motor for cytoplasmic streaming and transport of organelles and vesicles in pollen tubes. Recently, microtubule-based molecular motors (kinesins) were also identified and shown to be responsible for a low-speed transport of some organelles and vesicles in pollen (Yokota and Shimmen, this volume).

Myosin is composed of heavy and light chains and is grouped into at least 18 classes on the basis of the primary structure of the heavy chain genes in eukaryotes (Seller 2000; Berg et al. 2001). From plant cells, three classes of myosins, VIII, XI and XIII, have been identified (Reichelt and Kendrick-Jones 2000; Reddy and Day 2001). To date, class XIII myosin has been found only in green alga *Acetabularia* and myosin VIII has not yet been reported in pollen. In *Arabidopsis*, there are four subclasses of myosin VIII, while myosin XI forms a large gene family with at least 13 isoforms classified into three subclasses, MYA1, MYA2 and MYA3.

A subclass of myosin XI has been biochemically isolated from pollen (Yokota et al. 1999a; Shimmen and Yokota 2004). This 170-kDa myosin was composed of a 170-kDa heavy chain and CaM as the light chain. The sliding velocity of actin filaments induced by this myosin in vitro was comparable to the velocity of cytoplasmic streaming observed in living pollen tubes (Yokota and Shimmen 1994). The 170-kDa polypeptide with similar antigenicity to lily 170-kDa myosin heavy chain was also present in tobacco pollen and vegetative cultured cells, and located around surfaces of organelle with various sizes (Yokota et al. 1995, 1999b). These results demonstrated that this myosin is a motor for generating motive force in pollen tube cytoplasmic streaming and that Myosin XI is a general molecular motor for streaming and organelle transport also in vegetative cells (Shimmen and Yokota 2004). The motile activity and actin-activated ATPase activity of 170-kDa myosin were suppressed

reversibly by micromolar concentrations of Ca^{2+} through CaM light chain (Yokota et al. 1999a). When Ca^{2+} concentrations were elevated up to 10^{-5} M, the CaM light chain was dissociated from heavy chain but this phenomenon is not physiologically relevant, because $[Ca^{2+}]_c$ concentration is not so high in pollen tubes (Hepler et al., this volume). Taken together, these properties explain why an organized streaming is not observed in the apical region, it is doubled blocked by a Ca^{2+}-inhibited myosin activity and actin fragmentation.

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The Microtubular Cytoskeleton in Pollen Tubes: Structure and Role in Organelle Trafficking

Giampiero Cai (✉) · Mauro Cresti

Dipartimento Scienze Ambientali "G. Sarfatti" – Università degli Studi di Siena,
Via Mattioli 4, 53100 Siena, Italy
cai@unisi.it, cresti@unisi.it

Abstract Microtubules are a fundamental component of plant cells, in which they achieve many critical functions. In pollen tubes, however, their specific role remains unsolved and ambiguous. Microtubules are extremely abundant in the pollen tube and are undoubtedly important in critical processes like the transport of sperm cells. Recent advances have also shown a dynamic interaction with pollen tube organelles and a low speed translocation suggesting that microtubules are not strictly essential in the cytoplasmic streaming but rather in the regulation of such process. Here we focus on the organization of microtubules and on their putative role in the transport of pollen tube organelles. We will discuss the model of functional cooperation between microtubules and actin filaments and adapt it to the pollen tube system.

1 Introduction

Microtubules are a fundamental component of the cytoskeleton in all eukaryotic cells operating in the intracellular positioning and dynamics of organelles and molecules. Microtubules are the main scaffold along which chromosomes separate during cell division, representing the tracks for the precise transport of organelles and vesicles and constituting the central part of specialized structures like cilia and flagella. The organization of microtubules in plant cells is rather different from the one typically found in an animal cells, as four distinct arrays of microtubules are characteristically present throughout the cell cycle (Goddard et al. 1994). Unlike somatic cells, the pollen tube is a cell that continuously grows but does not divide. Consequently, we can only distinguish the interphase microtubule array (Fig. 1), which shares some parallelism but also exhibits a number of differences compared with somatic cells. In pollen tubes, microtubules are organized as thick bundles in the base region of the tube, which progressively shift into thin bundles or likely into single microtubules in the subapical region (Del Casino et al. 1993; Derksen et al. 1985; Lancelle et al. 1987; Pierson et al. 1986; Raudaskoski et al. 1987). Their presence in the apical domain is still questionable. Detection of microtubules in the tube apex may be consequential to fixation artifacts, but it may also reflect the dynamicity that characterizes the growth

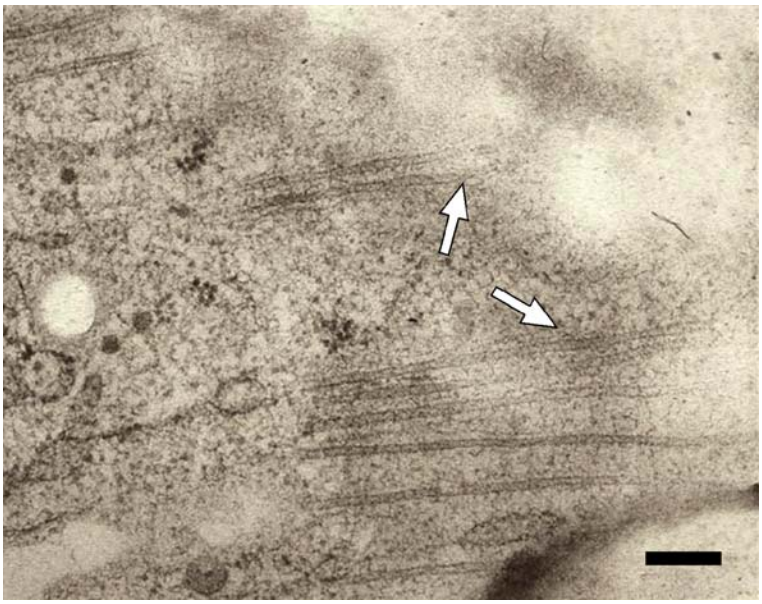

Fig. 1 Microtubules in the pollen tube of tobacco. The electron micrograph after freeze-fixation shows the organization of microtubules in the cortical region of tobacco pollen tubes. Arrows indicate microtubules that are positioned parallel to each other and according to the elongation axis of the cell. Bar: 200 nm. Courtesy of Fabrizio Ciampolini/Claudio Milanesi

region. In addition, microtubules were reported to be present in the apex only when the pollen tube changes the growth direction (Foissner et al. 2002), suggesting a role in guidance and emphasizing a significant contribution under in vivo conditions. Although microtubules are likely involved in many functions during tube growth, this chapter will mainly focus on a specific aspect of the pollen tube microtubules, namely their involvement in the transport of organelles and vesicles.

2
The Structure of Microtubules in Pollen Tubes

2.1
Molecular Composition

Microtubules are composed of α- and β-tubulin subunits, two polypeptides with nearly identical molecular weight and amino acid sequence (McKean et al. 2001). Each subunit is represented by different isotypes, which are consequence of the expression of different genes or of selective posttranslational

modifications. The *Arabidopsis* genome contains several tubulin genes, at least six different α-tubulin genes and nine β-tubulin genes (Kopczak et al. 1992; Snustad et al. 1992). Consequently, the molecular composition of microtubules may differ according to specific tissues. For example, one α-tubulin isotype of *Arabidopsis* is preferentially expressed in pollen (Carpenter et al. 1992). The same concept can be applied to β-tubulins. In carrot cells, at least six β-tubulin isotypes can be discriminated by 2D-electrophoresis. The isotype β4 appears in immature and mature stamens and becomes the predominant isotype in mature pollen. Other isotypes (β1 and β3) are conversely absent from pollen (Hussey et al. 1988). In rice, seven β-tubulin genes are mainly expressed in leaves, while TUB8 is preferentially expressed in anthers and in mature pollen (Yoshikawa et al. 2003). The presence of one anther-specific β-tubulin gene suggests its importance during anther and pollen development or at some stage of pollen tube growth. The TUB9 (β-tubulin) gene of *Arabidopsis* is equally expressed in floral tissues, with the highest levels of expression observed in pollen, pollen tubes and ovules (Cheng et al. 2001). A characteristic α-tubulin gene is restricted to the male gametophyte of sunflowers and few other species (Evrard et al. 2002). Pollen tubes are also characterized by high levels of tubulin (and actin) transcripts, which could be generated during pollen tube growth (Sorri et al. 1996). These results consequently suggest that the development and functioning of the male gametophyte may require the presence of specific α- and β-tubulin genes and proteins, although the differential expression does not always provide definite functions.

Tubulin is also posttranslationally modified to achieve complete functioning. Both acetylated and tyrosinated forms of α-tubulin have been detected in tobacco pollen tubes using specific antibodies. Tyrosinated α-tubulin was found in the apical domain of pollen tubes and, following elongation, along the base domain (Del Casino et al. 1993). In both cases, the staining pattern was distinct from the classical filamentous organization, suggesting that the tyrosinated form of α-tubulin does not distribute uniformly along microtubules. Tyrosinated α-tubulin may be used to mark specific domains of the microtubule population. However, whether such domains correspond to more or less stable microtubules is not determined. Acetylated α-tubulin was detected in the generative cell at different developmental stages (in the kinetochore fibers, in the polar regions and in the phragmoplast) (Astrom 1992). As the generative cell microtubules have been suggested to be more stable that the vegetative microtubules, acetylated α-tubulin may correlate with and consequently mark the arrays of more stable microtubules. The posttranslational acetylation seems to concern with the most abundant isoform of pollen tubulin, the α3, which is also considerably polyglutamylated. In addition, tyrosination of the tubulin isoform α6 seems to be pollen-specific (Wang et al. 2004). Tubulin from *Malus domestica* pollen is also modified by transglutaminases through the addition of polyamines (Del Duca et al. 1997). As

the transglutaminase-catalyzed reactions can interfere with the binding of molecular motors to cytoskeletal filaments (Kim et al. 1998), the posttranslational addition of polyamines may be used to inactivate the cytoskeletal-based transport of organelles in pollen.

All together, these evidences suggest that the assembly of microtubules in pollen requires the expression of specific tubulin genes (in addition to those ubiquitously expressed) as well as the posttranslational modifications of both subunits. Why all these differentially-expressed genes and their relative post-translational alterations are necessary and how those different isotypes interact with other cell components is actually unknown. One simple hypothesis is that the growth of pollen tubes proceeds through the assembly of different microtubule subsets, such as those around the generative cell or at the tube apex.

2.2 Sites of Origin

In plant cells, the exact places from which microtubules originate have always been a matter of discussion. The absence of centrioles (a specific marker of microtubule-organizing centers in animal cells) never allowed researchers to identify precisely where microtubules initiate in plant cells. In interphase cells, microtubules are mainly cortical, suggesting that the plasma membrane should be involved in the organization of microtubules. Clearer evidence came from the discovery of γ-tubulin (a critical component of the microtubule-organizing complex) in plants and its association with membranes (Bo et al. 1994; Drykova et al. 2003). The current model predicts that plant microtubules are nucleated by distinct but flexible centrosome-like structures located in different cell regions, such as the nuclear surface and the plasma membrane (Chan et al. 2003). Unlikely somatic cells, pollen tubes grow by local expansion at the tube apex and microtubules are arranged along the growth axis. Furthermore, γ-tubulin is found all along the microtubules with no predominant distribution in specific cell regions (Palevitz et al. 1994). Thus, in pollen tubes the putative microtubule-nucleation sites may be uniformly distributed. On the contrary, another putative centrosomal protein (p77) seems to accumulate in the tube apex (Cai et al. 1996), suggesting that the tip domain may be important in the assembly of new microtubules. When pollen tube microtubules are depolymerized by drug or cold treatment, their recovery initiate at the cell periphery (Åström et al. 1991; Heslop-Harrison and Heslop-Harrison 1988) but recovery from treatment with calyculin A indicated no preferential sites for the newly growing microtubules (Foissner et al. 2002). A hypothesis to explain these data is that the tip domain of pollen tubes may function similarly to the nuclear surface of somatic cells, which is considered the main plant microtubule-organizing centre. As plant γ-tubulin is distributed all along the microtubule length, it

may be more directly involved in microtubule dynamics rather than in their nucleation (Schmit 2002). Consequently, pollen tube microtubules could be initially assembled as single short (and possibly highly dynamic) structures in the tip domain by protein complexes containing p77; if the tube apex is converted into a more stable domain during growth, microtubules could be then stabilized and further elongated in the cell cortex through the incorporation of γ-tubulin subunits. The stabilization of microtubules could involve their assembly into bundles by putative microtubule-associated proteins and their lateral binding to the cell membrane through microtubule-membrane proteins, like p161 (Cai et al. 2005). The role of actin filaments and of the cell wall in the stabilization of microtubules is unidentified. This model is summarized in Fig. 2.

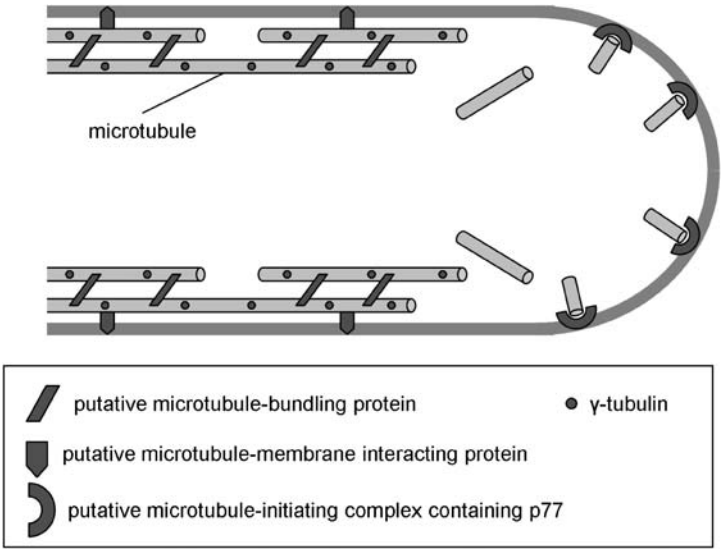

Fig. 2 Sites of origin and the organization of microtubules in the pollen tube. Microtubules may originate in the apical and subapical region of the pollen tube through the activity of nucleating complexes containing the centrosome-like protein p77 and γ-tubulin. In this region, microtubules may be single, short and highly dynamic structures, a precondition for a cell region characterized by rapid changes in ion and molecule composition. The stabilization of nascent microtubules may occur in the subapical region and be dependent on the activity of different molecular complexes containing γ-tubulin. Further stabilization and bundling of microtubules may be then generated by microtubule-associated proteins that connect microtubules to the plasma membrane (like p161) or to each other. Association of microtubules with actin filaments may occur through the activity of proteins like p190 (Igarashi et al. 2000) but it is speculative and simply based on the co-localization of the two cytoskeletal systems. How microtubules may eventually influence the organization of actin filaments (and vice versa) is unknown. Objects are not drawn to scale

3
Proteins that Interact with Microtubules

3.1
Microtubule-associated Proteins

Microtubules achieve most of their functions using a set of specialized proteins (microtubule-associated proteins, or MAPs), which change microtubules from a static filament into a dynamic structure. MAPs are a wide class of proteins that allow microtubules to control the rate of polymerization–depolymerization and to interact with each other and with other cell structures. The research in the plant MAP field is relatively recent in comparison with the animal counterpart. This is essentially due to the relatively low abundance of MAPs in the plant cell and to the lack of a model cell like neurons. Standard biochemical approaches have allowed the identification of a small number of proteins that fit the criteria for MAPs (Sedbrook 2004) but genetic and molecular studies have significantly extended the identification of plant MAPs. For example, MOR1 is a microtubule organizing protein originally identified in *Arabidopsis* using temperature-sensitive mutants. MOR1 is the plant version of an early family of MAPs that is essential for the organization of cortical microtubules (Whittington et al. 2001). Another example of genetically-characterized MAPs is the MAP65 family (Hussey et al. 2002).

Evidences for MAPs in pollen tubes are even scarcer. Using a method based on the taxol-dependent polymerization of endogenous microtubules, Tiezzi et al. (1987) have initially identified pollen tube proteins that interact with microtubules. Apart from this initial study, no further evidences have been obtained despite the good qualities of pollen tubes as a model system; microtubules show different organization levels relating to the different stages of pollen tube growth, as the distribution and structure of microtubules radically change during pollen hydration and germination (Tiwari and Polito 1990). All of these dynamic arrangements imply the presence of MAPs, which could control the assembly and interaction of microtubules with other cell components. Other structural evidences for the presence of MAPs are the spatial association of microtubules with actin filaments (Lancelle and Hepler 1991) and with the endoplasmic reticulum (Hepler et al. 1990). Recently, two proteins with MAP-like properties have been identified in the pollen tube of tobacco on the basis of the immunological cross-reactivity with antibodies to plant MAPs (Cai et al. 2005). These proteins interact with both animal and plant tubulin, can control the rate of tubulin assembly and localize both in the vegetative and generative cytoplasm. The most striking feature of those proteins is their interaction with the plasma membrane, which suggests their involvement in the association of microtubules with the cell membrane. In this context, the physical association between microtubules

and the plasma membrane would be a prerequisite for the correct modeling and shaping of the pollen tube. Microtubules would provide the scaffold for the cylindrical shape of the pollen tube, most likely in association with the cell wall components.

The identification of MAPs and the characterization of their activity are also important to understand how the assembly and dynamics of microtubules are regulated. The phosphorylation of both or either tubulins and microtubule-interacting proteins seems critical in the regulation of microtubule organization (Foissner et al. 2002) and could occur through the alteration of associated proteins like in other cell types (Gong et al. 2000).

3.2 Kinesin-like Proteins

Kinesins are microtubule-based motors that play different roles in cells and are coded by a large gene family whose sequence homology is sometimes restricted to small domains. The most important feature of kinesins is their ability to interact with and move along microtubules using the energy of ATP hydrolysis. The functions of kinesins are broad and include the transport of organelles and the relocation of mRNAs (Hirokawa and Takemura 2004). Kinesins are grouped into distinct subfamilies that have been recently reviewed and standardized on the basis of 14 family designations (Lawrence et al. 2004). Screening of the entire *Arabidopsis* genome has revealed more than sixty kinesin-like proteins (Lee and Liu 2004; Reddy and Day 2001) although it is not clear whether some of these genes are pseudogenes. The number is nevertheless huge if compared with the kinesin gene family in other genomes. It is difficult to assign a function to plant kinesins simply based on the specific subfamily to which they belong, but it is possible that plant kinesins perform different tasks from their animal counterparts belonging to the same subfamilies. However, most of the plant kinesins are expected to have a role in the assembly of the mitotic spindle and in the organization of the cortical microtubule array (Liu and Julie Lee 2001). Few indications support a role for plant kinesins (and generally for microtubule motors) in organelle transport and most of these evidences are indirect (Krishnakumar and Oppenheimer 1999; Sato et al. 2001; Van Gestel et al. 2002). Remarkably, the pollen tube is likely the plant cell on which we have more information on the interaction between kinesin-like proteins and cell organelles (Cai et al. 2001).

Kinesin-like proteins were first identified in the pollen tube of tobacco using a monoclonal antibody raised against the heavy chain of bovine brain kinesin (Tiezzi et al. 1992). The antibody allowed the identification of a $\sim$ 100 kD-polypeptide that binds to microtubules. Further biochemical analysis revealed that the polypeptide has a microtubule-dependent ATPase activity and a ATP-dependent microtubule-binding ability (Cai et al. 1993).

The polypeptide was localized by immunofluorescence microscopy in the apex and, more faintly, in the flanks of pollen tubes. The antibody also cross-reacted with one polypeptide of similar molecular mass in hazel pollen (Liu et al. 1994) that localized in association with Golgi vesicles. These preliminary evidences indicated that pollen vesicles could have a microtubule-dependent motor on their surface but did not provide support for the movement of vesicles along microtubules. Remarkably, a comparable localization pattern was found in the pollen tube of gymnosperms in which a different antibody to kinesins stained the tube apex (Terasaka and Niitsu 1994).

The use of a different pan-kinesin peptide antibody revealed the presence of kinesin-like proteins in the generative cell of tobacco and, partially, in the vegetative cytoplasm (Liu and Palevitz 1996). This motor was supposed to have a role in the formation of the sperm cells in tobacco.

A further step in our understanding of the role of kinesins in pollen tubes was indirect. One *Arabidopsis* gene, ZWICHEL (ZWI), is critical in the process of trichome morphogenesis and is a member of the kinesin superfamily. Double mutants in the ZWI and SUZ genes (SUZ is an extragenic suppressor of zwi mutations) exhibited a male-sterile phenotype because of defects in pollen tube germination and growth (Krishnakumar and Oppenheimer 1999). These observations indicated a role for the kinesin-like gene ZWI in pollen germination and pollen tube growth but did not demonstrate that pollen tube organelles move along microtubules. This came with the identification of 80 and 90 kD proteins called ATP-MAPs which were identified based on their selective binding to microtubules (Cai et al. 2000). In addition to be a microtubule-dependent ATPase, the 90-kD ATP-MAP is able to move microtubules on a glass surface, is recognized by a pan-kinesin antibody and localizes in the cortical region of pollen tubes (but not in the apical domain) in association with membrane-bounded organelles. The relatively low speed of microtubule gliding suggested that the 90-kD kinesin-like protein is not involved in the fast transport of pollen tube organelles. The relative distribution of these potential microtubule motors in the pollen tube is illustrated in Fig. 3. A second critical evidence was the observation that isolated pollen tube organelles move along microtubules (Romagnoli et al. 2003). The trafficking ability involves different classes of organelles, from relatively low-density vesicles to high-density mitochondrial fractions and is dependent on ATP but independent on soluble factors. These data suggest that pollen tube organelles have microtubule-dependent motors tightly associated with their surface. Again, the motility speed was much lower than the cytoplasmic streaming of pollen tubes. The organelle fraction showing the higher transport rate contains a membrane protein of ~ 105 kD that binds to microtubules in a ATP-dependent manner and is also recognized by anti-kinesin antibodies. This protein is consequently a likely candidate to move organelles in the pollen tube.

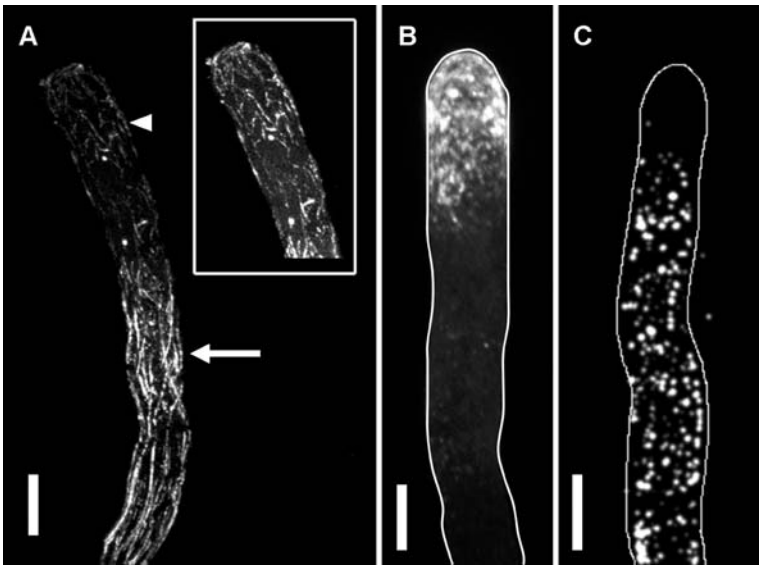

Fig. 3 Distribution of microtubule-dependent motors in the pollen tube of tobacco. Immunolocalization of microtubules (**A**) and of two microtubule-dependent motors (**B**, **C**). All images are from chemically-fixed materials. **A** Microtubules are organized as bundles positioned along the elongation axis of the pollen tube. The base region of the pollen tube contains thicker bundles (*arrow*), whereas the apical domain contains thin fibrillar elements or no microtubules at all (*arrowhead*). In the *inset* in A, the *grayscale levels* have been changed to emphasize the faint (and possibly dynamic) microtubule cytoskeleton in the apex (the presence of microtubules in the apical domain is still speculative). **B** The anti-kinesin k71s23 (Tiezzi et al. 1992) yields a faint but distinct staining pattern in the tube apex, suggesting the presence of kinesin-like proteins in association with the secretory vesicles. **C** The antibody MMR44 (Cai et al. 2000) stains punctuate structures in the pollen tube with the exception of the apical domain, suggesting the association of different kinesins with pollen tube organelles. Bars: 15 μm. **A** is a courtesy of Cecilia Del Casino and **C** is a courtesy of Antonio Tiezzi and Elisa Ovidi

3.3 Dynein-like Proteins

The presence of dynein or dynein-like proteins in plants is still debated. The last years have seen contradictory manuscripts on this topic. The analysis of the *Arabidopsis* genome has shown that dynein-like sequences are missing (Lawrence et al. 2001), suggesting that plants may have evolved a different or alternative mechanism for the minus end-directed movement of organelles along microtubules or that plants may simply not possess this type of transport. Conversely, examination of the whole genome shotgun sequence for rice indicated the presence of four dynein heavy chains (King 2002). This finding may indicate that the absence of dynein sequences in the *Arabidopsis* genome

is not a general trait and that plants may really use dynein-based mechanisms for the intracellular transport. Another interpretation for the missing of dynein-like sequences in *Arabidopsis* may arise from the large number of reported kinesin-like proteins; twenty-one genes coding for minus end-directed kinesins have been found in the *Arabidopsis* genome (Lee and Liu 2004), suggesting that the mechanochemical work performed by dynein may be as much efficiently done in plants by kinesins. Further data supporting the existence of plant dyneins is the biochemical identification of dynein-like polypeptides in tobacco pollen tubes (Moscatelli et al. 1995) and their apparent association with pollen tubes organelles (Moscatelli et al. 1998). In addition, a small fragment of a gene coding for the tobacco dynein heavy chain was isolated from genomic DNA and from a cDNA library of pollinated styles (Scali et al. 2003). The fragment has a very high homology with the *Chlamydomonas dhc*1 gene for 1-alpha dynein heavy chain and contains the first P-loop consensus motif, a region highly conserved in all dyneins.

4
One Choice or Two for Organelle Movement?

What can we learn from these few reports? The massive transport of organelles in plants (designated as "cytoplasmic streaming") is supposed to occur on actin filaments and to be dependent on myosins (Chapter VII). Recent advances on the study of organelle transport in plant cells have made use of modern technologies to dissect all the molecular components that take part into the process. The movement of plant cell organelles along actin filaments is now clearly established: the photorelocation of chloroplasts, the positioning of mitochondria, the transport of peroxisomes and the movement of Golgi bodies along networks of endoplasmic reticulum are all examples of how plant cell organelles interact dynamically with the actin cytoskeleton (Wada and Suetsugu 2004). In opposition, the role of microtubules and of microtubule-dependent motors in plant organelle trafficking has always been misleading. For example, the shaping and motility of the endoplasmic reticulum in plants is known to be affected by cytochalasin D but is relatively independent on microtubule-affecting drugs, such as colchicine or oryzalin (Knebel et al. 1990). However, microtubules have a critical role in cell morphogenesis and maintenance of cell polarity. Genetic and pharmacological studies indicated that the role of actin filaments and microtubules is more or less the same in tip-growing and diffuse-growing cells: while microtubules appear to be important for establishing and maintaining the cell polarity, actin filaments move the materials necessary for growth to particular sites. To sum up, microtubules say how to organize a cell and where to transport materials whereas actin filaments do the transport (Mathur and Hulskamp 2002). Examples of this putative function of microtubules are many. In rhizoids,

microtubules have a central role in determining the cell polarity while the actin cytoskeleton is responsible for the motile processes (Braun and Sievers 1994). In trichome morphogenesis, disruption of actin networks does not affect the establishment of polarity, while the depolymerization of microtubules inhibit cell polarization, causes trichome cells to swell and stops the focused growth (Szymanski et al. 1999). The depolymerization of microtubules in root hairs causes the loss of directionality of growth and the formation of many autonomous growth points (Bibikova et al. 1999). In *Medicago truncatula* root hairs, microtubules form a specific subset, called endoplasmic microtubules, which is present all through the subapical cytoplasmic region but progressively disappears during growth arrest, in full-grown root hairs and after treatment with oryzalin (Sieberer et al. 2002). The characterization of *Arabidopsis* mutants with growth defects in hypocotyls cells allowed the identification of a small protein (SPR1) that colocalizes with cortical microtubules (Nakajima et al. 2004). This protein is highly expressed in tissues showing rapid cell elongation, suggesting that plant-specific microtubule-associated proteins are necessary for the anisotropic growth of elongating cells. In pollen tubes of *Picea abies*, microtubules and actin microfilaments form a dense matrix corresponding to the direction of cell elongation; depolymerization of microtubules causes the pollen tube to stop elongation and to branch while disturbance of actin filaments stops tube growth and blocks germination (Anderhag et al. 2000). Consequently, microtubules are important in promoting tip extension in conifer pollen tubes, likely by controlling the positioning of organelles in the tip and by mediating the organization of actin filaments (Justus et al. 2004).

The role of microtubules in the angiosperm pollen tube is more difficult to decipher. The use of specific antagonists initially showed that microtubules are not important for organelle transport (specifically, for the cytoplasmic streaming) and for tube growth (Heslop-Harrison et al. 1988). However, comparable studies with different microtubule antagonists revealed that microtubules might take part in different aspects of the pollen tube growth. For examples, the in vivo growth (Joos et al. 1995), the cell polarity during in vitro growth (Joos et al. 1994), the pulsatory growth (Geitmann et al. 1995) and the positioning of organelles (He et al. 1995) are all affected by anti-microtubule drugs. The most dramatic effect of microtubule depolymerization in the pollen tube concerns the motility of both generative cell and vegetative nucleus. The application of oryzalin on tobacco pollen tubes significantly reduces the motility rate of the male germ unit, suggesting that microtubules have a critical though not complete role in this motor activity (Astrom et al. 1995). In addition, the movement of the generative cell is unrelated to the synthesis of the callose plugs, which indicates that microtubules may not take part in their production (Laitiainen et al. 2002). All of these data suggest that microtubules take part into distinct processes and, consequently, that they interact with several cellular compartments. The precise functions

they have are nevertheless uncertain, and uncertain is how they interact with each other and with other cell components and what proteins mediate such interactions. For example, the finding that oryzalin treatment reduces the motility rate of the generative cell may suggest that either microtubule-dependent motors are present at the generative cell surface or the correct organization of actin filaments around the generative cell is dependent on the proper architecture of microtubules. Alternatively, microtubules may simply indicate to the generative cell where to move, leaving the motion force to actin filaments. Consequently, a conclusion on the role of microtubules in the pollen tube needs supplementary methodical investigations with different techniques in addition to drug studies.

The relationships between microtubule-dependent motors and organelle transport are more difficult to understand. The cytoplasmic streaming depends essentially on actin filaments (Chapter VII) but the importance of the coordination between the actin- and microtubule-based transports must be emphasized. Although most reports state that organelle transport relies on actin filaments, data on the presence of microtubule-dependent motors and the results of in vitro motility assays (Romagnoli et al. 2003) suggest that some kind of microtubule-based movement should take place in the pollen tube. The challenge is now to make sense of this specific motility in the context of the pollen tube growth. For this purpose, we can compare what is already known in other cell types. Generally, the movement of cytoplasmic organelles in eukaryotic cells is recognized to be based on both microtubules and actin filaments and on their dependent motor proteins. This model is called "functional cooperation" and is supported by several data from different cell types (Goode et al. 2000). Briefly, the model assumes that cell organelles have motor proteins of different families associated with their surface; furthermore, the model suggests that the correct transport and positioning of organelles is the result of two distinct processes that do not exclude each other. The first process consists of the transport of organelles throughout the cell and is dependent on the sequential activity of microtubule- and actin-based motors. In nerve cells, for example, organelles are transported along the axon using microtubules and kinesins while the final delivery in the synapse is mediated by actin filaments and myosins (Brown et al. 2004). The second process consists of the correct transport and positioning of organelles in non-nerve cells and is dependent on the simultaneous activity of motors from different families. As an example, the proper positioning of organelles in melanophores is maintained by the steady activity of the motor forces of kinesin, dynein and myosin (Lambert et al. 1999). Although nerve cells and melanophores are distant from plant cells, a similar kind of cooperation has been recently suggested to occur in characean internodal cells (Foissner 2004), in which the transport of mitochondria could occur along both microtubules and actin filaments according to the different pH of specific cell regions.

The extension of this model to the pollen tube may be hazardous. However, if we sum up all the data on motility and motors, we can develop one model that takes into account the role of actin filaments, microtubules and their dependent motors (kinesin and myosin). The model is schematically summarized in Fig. 4. In theory, it is possible to distinguish at least two different regions of the pollen tube in which the functional cooperation could efficiently occur: the base region and the apical region. In the base region, transport of organelles occurs according to the standard scheme of cytoplasmic streaming and is likely to be mostly dependent on actin filaments and myosins. In this particular region, the slow motion mediated by

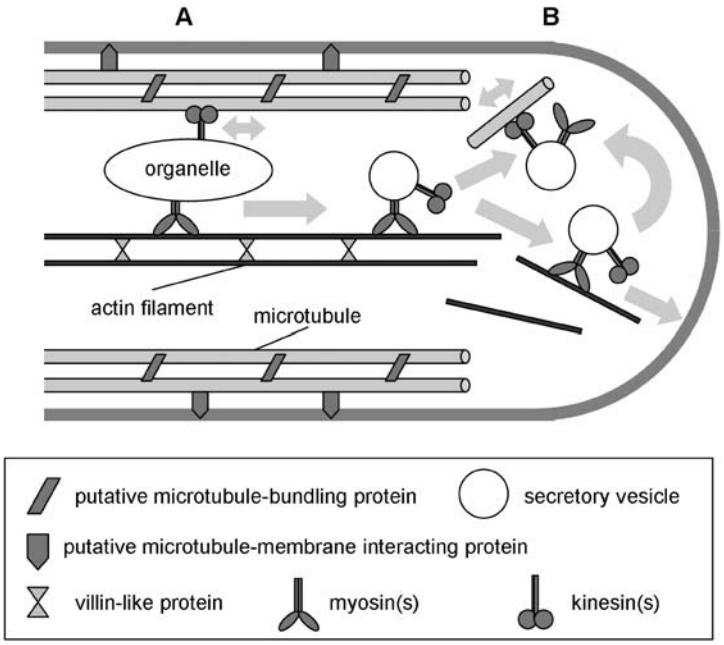

Fig. 4 Examples of functional cooperation between microtubule- and actin filament-dependent motors in the pollen tube. Microtubules may cooperate in two ways with actin filaments in promoting the correct positioning of organelles. In the first case, microtubule- and actin filament-dependent motors (kinesins and myosins) are used simultaneously **A** in order that the net movement of each organelle derives from the equilibrium of both motors' activity. The *long grey arrows* indicate that the organelle has a preferential movement along actin filaments, whereas the *short bidirectional arrows* indicate that microtubule-dependent motors may act as a regulator of the actin-based movement. In the second case (**B**), motors are used sequentially to promote the correct delivery or to maintain the position of vesicles in the tube apex. *Arrows* indicate the same type of movement described previously. In order to complete the scheme, other proteins have been drawn (e.g. the villin-like protein), which forms actin bundles, and some microtubule-binding proteins. Objects are not drawn to scale

microtubule-dependent motors could serve to regulate the trafficking of organelles. Consequently, motors from different families should work together to ensure a correct transport of organelles. In the apical region, accumulation of vesicles requires an intact (and dynamic) actin cytoskeleton, which serves as tracks for the myosin-dependent movement of vesicles. In this context, the role of microtubules and dependent motors may be consecutive or complementary to the one of actin filaments. Microtubules and kinesins may be used in processes related to tip growth, like exocytosis, endocytosis, the focusing of secretory vesicles (Bi et al. 1997), or the maintaining of specific proteins in the apical plasma membrane. Alternatively, their role could be critical when the pollen tube grows within the style and consequently undergoes regulation by the pistil.

5
The Organization of Microtubules in Relation with Pollen Tube Growth

There are two aspects of the pollen tube physiology where the function of microtubules is still elusive: cell shaping and cell signaling. The former is known to depend on the organization of cortical microtubules, which in turn influences the pattern of cellulose deposition and then how a cell expands (Wasteneys 2004). The stability of cortical microtubules is a function of their orientation, because divergent microtubules within the array depolymerize in few minutes. The dynamics of microtubules in the cortex is likely dependent on the activity of different proteins that regulate their stability, the formation of bundles, the interaction with other cell structures, the relative sliding to each other and their interaction with the cellulose-synthase complex (Sedbrook 2004). All these concepts are partially adaptable to the pollen tube. In fact, pollen tube microtubules are aligned along the growth axis of the tube, while they are usually disposed transversally to the expansion axis in somatic cells. This divergent way to organize microtubules weakens the similarity of their role between the pollen tube and somatic cells and thus questions if microtubules control the shape of the pollen tube. Cell morphogenesis is a biological puzzle known to depend on two important events: the accumulation of extracellular material and the mechanical deformation of the cell surface (see Chapter IX).

The second critical aspect is cell signaling. The pollen tube is a cell that constantly grows and changes its direction following the interactions with other cells; however, we ignore how external signals influence the organization of microtubules and if (or how) alterations in their structure may affect the movement of organelles and the organization of the cell wall. The cascade of intracellular signals following the interaction of the pollen tube surface with extracellular hints has effects on many of the cellular activities in the pollen tube. It is likely that the microtubule cytoskeleton of the pollen tube

responds to this signaling cascade and organizes accordingly. MAPs are good candidates to mediate such re-organization activity. In this context, proteins analogous to phospholipase D are candidates to convert the extracellular signals into signals that microtubules can recognize (Chapter VI). Activation of phospholipase D affects the organization of plant microtubules by releasing them from the plasma membrane and by partial depolymerization (Dhonukshe et al. 2003). Hypothetically, during the in vivo growth of the pollen tube, external signals (after conversion into intracellular messengers that control the organization of microtubules) may regulate the transport rate of the generative cell and vegetative nucleus and the "concentration" of organelles in specific domains of the pollen tube. A nicely and intriguing though speculative hypothesis is that microtubules and actin filaments may regulate and reorganize after receiving external information. The reorganization of the cytoskeleton may then influence the way organelles distribute in the pollen tube cytoplasm and, in turn, influence the growth direction and the polarity of the pollen tube. This hypothesis assumes that signaling molecules bind to microtubules and mediate the activity of different environmental triggers (such as cold, osmotic stress and pathogens) (Wasteneys 2004). The consequence of this theory is that actin filaments are the operative force that guides organelle movement in the pollen tube, while microtubules may appropriately contribute to dispose organelles at their right place.

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The Architecture and Properties of the Pollen Tube Cell Wall

Anja Geitmann[1] (✉) · Martin Steer[2]

[1]Institut de recherche en biologie végétale, Département de sciences biologiques, Université de Montréal, 4101 Sherbrooke est, Montréal, Québec H1X 2B2, Canada
anja.geitmann@umontreal.ca

[2]School of Biology and Environmental Science, University College Dublin, Dublin, Ireland
martin.steer@ucd.ie

Abstract The pollen tube wall differs in both structure and function from walls of vegetative plant cells. Cellulose represents only a small portion of the cell wall polymers, so an organized microfibrillar system has not been identified yet. The initial wall, formed by secretion at the growing tip, is mostly composed of methyl esterified pectins. During cell wall maturation, concomitant with its translocation from apex to shank, these are demethylated by pectin methylesterase to yield carboxyl groups which have the potential to bind calcium ions, adding mechanical strength to the gel. Callose synthase activity is established close to the growing tip, and builds a callose layer beneath the fibrous pectic layer. The mature wall also contains proteins, arabinogalactan proteins and pollen extensin-like proteins. The mature wall is a cylinder that resists turgor expansion, but is stronger at the base than the tip due to the presence of the callose layer and the gelation of pectin polymers in the shank. Permeability of the wall is essential, to allow passage of both ions and sporophytic proteins that determine compatibility in many species. Influx of calcium ions affects the tip cytoplasm, especially the cytoskeleton, and oscillatory changes in these fluxes are involved in the "pulsatile" mode of growth. This process deposits extra wall material during the "slow" growth phase, which generates rings of increased density in the walls that can be readily seen with appropriate antibodies.

1 Introduction

The cell wall has multiple functions in pollen tube growth: physical control of cell shape, protection of the generative cell against mechanical damage, resistance against the turgor pressure and adhesion to the transmitting tissue. All of this makes the pollen tube cell wall a key feature in the process of fertilization. It is, therefore, not surprising that genes encoding for proteins related to cell wall biosynthesis and regulation are highly expressed in pollen (Becker et al. 2003; Honys and Twell 2003). While the volume of the cytoplasm does not change significantly during pollen tube elongation, the amount of total cell wall in the growing tube increases steadily. Therefore, the supply of cell wall precursors needs to be uninterrupted and rapid. To support this high

level of carbohydrate synthesis pollen tubes use both stored reserves which can include sucrose, starch, phytic acid and lipids, depending on the species (Jackson et al. 1982; Nakamura et al. 1980), and external sources of sugar.

2
Composition of the Pollen Tube Cell Wall

2.1
Cell Wall Polysaccharides

The cell wall of angiosperm pollen tubes has been studied extensively (Geitmann 1999; Taylor and Hepler 1997). The highly anisotropic growth behaviour of these cells is reflected in the non-uniform distribution of cell wall components. In the axial direction at least two regions have been identified, the approximately hemispherical growing tip and the cylindrical shank. In the radial direction, depending on the region, one or several cell wall layers can generally be distinguished. If a parallel is drawn with somatic cells, the wall laid down at the tip can be defined as the primary wall, while that formed in mature parts as secondary wall, because that part of the wall has stopped expanding. The temporal sequence of events in somatic cells is, therefore, transformed into a spatial pattern of features in the pollen tube. It is interesting to note that in isolated pollen tube protoplasts the sequence of cell wall deposition events seems to be reversed compared to the original cell. In pollen tubes pectins are laid down first, followed by callose deposition, whereas in isolated pollen tube protoplasts of olive the first cell wall polymer that reappears is callose, while label for pectins is only observed hours later (Majewska-Sawka et al. 2002). Instead of forming separate layers both cell wall components are intermixed in this case.

The tip region of angiosperm pollen tubes is generally characterized by a single wall layer (Lancelle and Hepler 1992). It is known to be mainly composed of newly synthesized pectins (Geitmann et al. 1995). This pectic layer can continue along the entire length of the pollen tube and forms the outer layer of the cell wall in the pollen tube shank, where a secondary wall layer is generally formed adjacent to the plasma membrane, or it can be gradually replaced by this secondary wall as seems to be the case in *Arabidopsis thaliana* (Derksen et al. 2002). From the outside, this wall has a porous appearance. The thickness of this primary wall is generally around 100 to 300 nm and in the transmission electron microscope (TEM) it appears distinctly fibrillar.

Pectins are galacturonate-rich, acidic polysaccharides, abundant in angiosperm pollen tubes. In the pollen tube tip region of angiosperms, pectins have a higher degree of methylesterification than in distal regions as shown with fluorescence label using monoclonal antibodies JIM5 and JIM7 (Figs. 1, 2) (Geitmann et al. 1995; Li et al. 1994). This de-esterification is

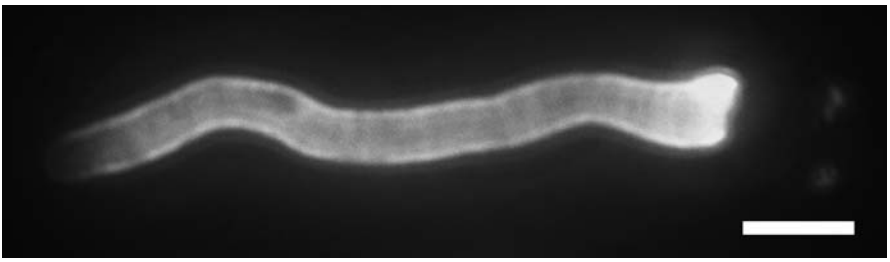

Fig. 1 Immunofluorescence label of *Solanum chacoense* pollen tube for pectins with low degree of methyl-esterification using monoclonal antibody JIM5. Label intensity is stronger at the shank of the tube than at the apex (located at the *left*). Bar = 10 μm. (Micrograph taken by Elodie Parre)

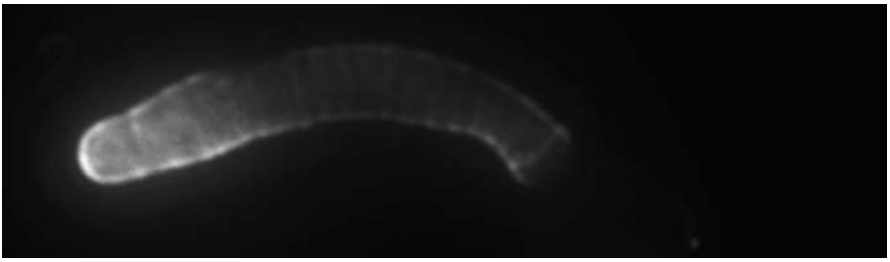

Fig. 2 Immunofluorescence label of *Solanum chacoense* pollen tube for pectins with high degree of methyl-esterification using monoclonal antibody JIM7. Label intensity is higher at the apex (located at the *left*). Bar = 10 μm. (Figure reproduced with permission from Parre and Geitmann 2005b)

caused by the activity of pectin methyl esterases secreted at the apex (Li et al. 2002). A distribution of methyl-esterified pectins similar to the one in angiosperms was observed in gymnosperm pollen tubes (Derksen et al. 1999a). In *Pinus*, no acidic pectin was found in the pollen tube except for the site where the inner intine of the pollen grain stretches over the pollen tube. Fluctuations in the growth rate cause the density of pectins to change along the longitudinal axis of the tube resulting in the appearance of more or less regularly spaced rings (Fig. 3). It should be noted that these fluctuations in pectin density are not accompanied by overall changes in cell wall thickness (Geitmann et al. 1995; Pierson et al. 1995), but after EDTA extraction periodic alternations of material with different material properties are revealed in the scanning electron microscope (Derksen, personal communication).

The "secondary" wall layer appears less electron dense and more homogeneous than the fibrillar outer layer (in TEM) even though stratification has been observed in certain cases (Kroh and Knuiman 1982). This inner layer consists mostly of callose as evidenced in the light microscope by staining with decolorized aniline blue. This stain shows that the polymer starts around

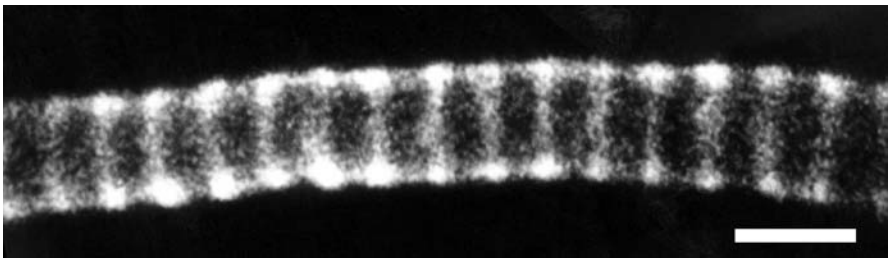

Fig. 3 Immunofluorescence label of *Nicotiana tabacum* pollen tube for pectins with low degree of methyl-esterification using monoclonal antibody JIM5. Label intensity shows regular fluctuations along the longitudinal axis of the pollen tube. The spatial frequency of the fluctuations corresponds to that of the growth oscillations. Bar = 10 μm. (Figure kindly provided by Mauro Cresti)

30 μm from the tip in *Nicotiana tabacum* (Ferguson et al. 1998). In *Papaver rhoeas* (Geitmann and Parre 2004) and *Arabidopsis thaliana* (Derksen et al. 2002), the first deposition of callose was observed closer to the tip which might be related to the lower growth rate in these species as discussed below. In petunia it was shown that the thickness of the callose lining increases gradually towards the base (Herrero and Dickinson 1981) whereas in poppy the level of label density did not change distal of 120 μm from the tip (Geitmann and Parre 2004). The use of monoclonal antibodies in TEM showed that callose is confined to the translucent inner layer of the cell wall (Geitmann et al. 1995; Meikle et al. 1991). Under certain circumstances the callose layer can thicken considerably. This is the case, for example, in self-incompatible pollen tubes that have stopped growing (Geitmann et al. 1995). In *Nicotiana* pollen tubes callose is a (1,3)-β-D-glucan with a few (1,6)-β-linked glycosyl branches (Rae et al. 1985). Unlike cellulose, callose is not organised as crystalline microfibrils, and is often described as amorphous. In addition to the inner wall layer, callose is also the main component of the callosic plugs that are formed at regular intervals in older tubes. These allow the living cytoplasm of the cell to be concentrated at the tip and separated from distal pollen tube regions that eventually degenerate. In *Pinus* pollen tubes no translucent inner wall layer is visible at the TEM level and no callose plugs are present (Derksen et al. 1999a).

In angiosperm pollen tubes, cellulose is present in exceptionally low amounts. Schlüpmann et al. (1994) calculated that in *Nicotiana alata* pollen tubes that had grown for at least 4 h, the cellulose content was only 10%, whereas 81% of the neutral polysaccharides was callose. The description of the subcellular localization of cellulose varies depending on the method used and species investigated. While many authors suggested that it is located in the outer layer because of the fibrillar appearance in the TEM of the latter (Shivanna and Johri 1985), immunolabel revealed cellulose to colocalise with callose (Fig. 4) (Ferguson et al. 1998). In grasses, on the other hand, it has

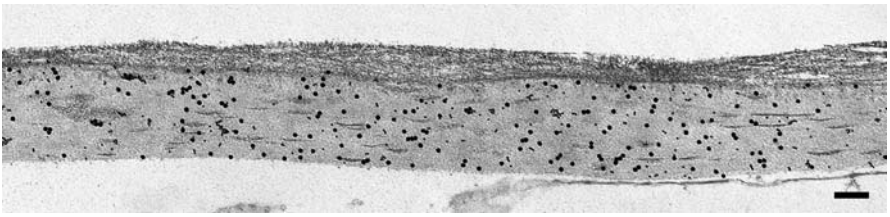

Fig. 4 Transmission electron micrograph of a longitudinal section in an older portion of a *Nicotiana tabacum* pollen tube. Black dots represent double gold-label with both cellobiohydrolase (CBHI) (20-nm gold) for cellulose and anti-(1,3)-β-D-glucan monoclonal antibody (30-nm gold) for callose. The colocalisation of label indicates that cellulose is located in the callosic inner wall layer of the pollen tube. (Figure reproduced with permission from Ferguson et al. 1998)

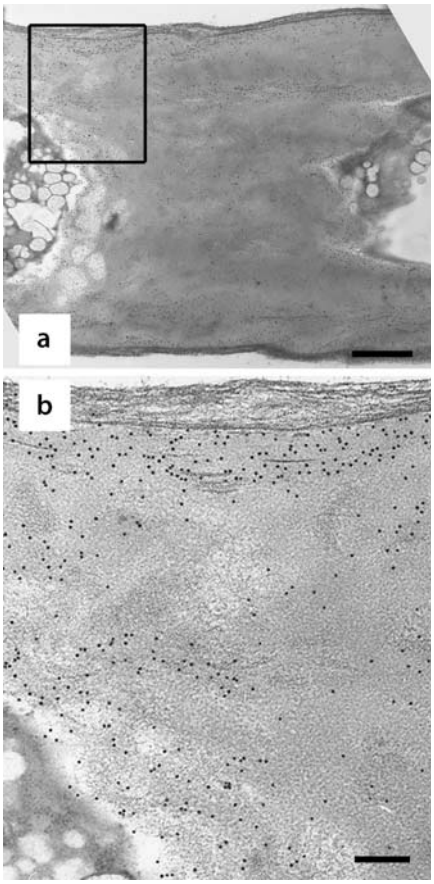

Fig. 5 Transmission electron micrograph of longitudinal sections of *Nicotiana tabacum* pollen tube plugs. **a**, **b** Labelled with CBHI for cellulose. Cellulose is associated with the periphery of the plug. Bars = 1 μm (**a**), 0.2 μm (**b**)

been reported that cellulose forms a third layer between callose and pectins (Heslop-Harrison 1987). In *Nicotiana*, cellulose was also found to be associated with the periphery of the callosic plugs (Figs. 5, 6) (Ferguson et al. 1998).

Unlike callose, cellulose is a crystalline component of the cell wall and therefore the orientation of microfibrils is potentially important for cell wall architecture. In *Petunia* pollen tubes, the direction of cellulose microfibrils seems to be random at the tip of the tube whereas in the shank they show a preferential angle of between + 45° and – 45° (Derksen et al. 1999; Sassen 1964) thus causing the appearance of birefringence. Sassen (1964) remarked also that on the inside of the wall close to the tip, cellulose microfibrils might

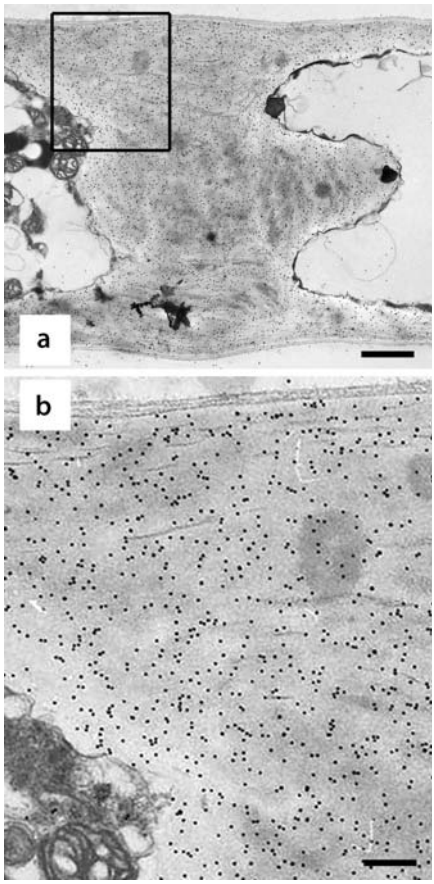

Fig. 6 Transmission electron micrograph of longitudinal sections of *Nicotiana tabacum* pollen tube plugs. **a**, **b** Labelled with anti-(1,3)-β-D-glucan monoclonal antibody for callose. Callose represents the main component forming the plug. (Figures reproduced with permission from Ferguson et al. 1998). Bars = 1 μm (**a**), 0.3 μm (**b**)

actually be oriented in transverse direction and suggested that this is the direction in which they were laid down. In angiosperm pollen, the tip is generally not or only weakly labelled for cellulose (Derksen et al. 1999; Ferguson et al. 1998), whereas in *Pinus* pollen tubes the amount of cellulose in the apex is relatively high (Derksen et al. 1999), which could be either a result or a cause of the low growth rate in these cells.

Taken together, the architecture of the pollen tube cell wall shows features typical of plant cells, such as primary and secondary layers. On the other hand it is rather unusual as it contains an unusually high percentage of callose compared to other plant cells. However, Steer and Steer (1989) pointed out that these data need to be interpreted with caution since almost all quantitative analyses were carried out in in vitro growing tubes. Plant cells are known to be able to switch between the production of cellulose and callose, for example upon wounding. It may be that the pollen tube surrounded by artificial liquid medium reacts by synthesizing a less permeable, callosic wall structure to maintain a particular environment. A first indication that this might be the case is the fact that pollen tubes grown in medium stiffened by agarose show lower quantities of callose than those grown in liquid medium (Parre and Geitmann 2005a). Further support comes from the comparison between in vitro and in vivo grown pollen tubes of *Arabidopsis* which indicates that the growth environment affects the cell wall. In this species the outer, fibrillar cell wall layer is absent from the basal parts of in vitro grown pollen tubes (Derksen et al. 2002), whereas it is present in the in vivo situation (Lennon and Lord 2000). Quantitative chemical and thorough histological analyses that compare in vitro and in vivo grown pollen tubes still remain to be done, however.

2.2 Polymer Networks

The physical properties of a cell wall are to a large degree determined by the capacity of its polymers to form networks. In pollen tubes at least two potential network formations are likely to be of importance: pectin gels formed through Ca^{2+} crosslinks and the cellulose-xyloglucan network.

The (partial) de-esterification of pectin increases its susceptibility to being crosslinked via Ca^{2+}-bridges, which is a gelation process (Carpita and Gibeaut 1993; Jarvis 1984). This ability has made pectins a widely used gelling agent. The gelation process increases the rigidity modulus of the pectin and thus the degree of esterification presumably influences the resistance of a pectic cell wall against mechanical stress. Given the large amounts of pectins present in the pollen tube cell wall and the non-uniform distribution of the degree of methyl-esterification, the gelling ability of this polymer is likely to be of importance for cellular mechanics and the regulation of the growth process.

While cellulose microfibrils have considerable tensile resistance parallel to their longitudinal axis, a stable three-dimensional network requires the inter-

action with hemicelluloses, in particular, xyloglucans, which form the linkages between the individual microfibrils (McCann and Roberts 1991; Whitney et al. 1995). In most plant cell types, extension growth under turgor pressure occurs by modulating this interaction of xyloglucan with cellulose microfibrils (Carpita and Gibeaut 1993). Knowledge about the presence of xyloglucans in pollen tubes is scarce, however. Polysaccharide linkage analyses of isolated pollen tube walls indicated that no significant amounts of xyloglucan are present in *Nicotiana* (Schlüpmann et al. 1994). On the other hand, immunolabel using CCRCM1, which recognizes a fucose-containing epitope found principally in xyloglucans marks the cell wall of pollen tubes of *Arabidopsis mur1* mutant (Freshour et al. 2003). Our own preliminary data show that the same antibody labels the pollen tube wall of other species (Aouar and Geitmann, unpublished data). While Freshour et al. found label primarily in the distal region of the tube, our data indicate the presence of CCRC-M1 label also in the apex. Further studies to exclude the possibility of non-specific cross-reaction of the antibody and to determine the role of xyloglucans for pollen tube cell wall architecture are presently underway.

2.3 Cell Wall Proteins

Pollen tube cell walls contain a considerable amount of protein. One group are the arabinogalactan proteins (AGPs), a class of high-molecular weight glycoproteins present in the majority of plants. They contain over 90% (w/w) carbohydrate and can be membrane-bound, wall-associated, or soluble (Nothnagel 1997) but their precise function is still largely unknown. Depending on the specificity of the antibody and on the plant species used, different locations of AGPs have been observed in pollen tubes. As a result, several possible functions have been suggested. Li et al. (1992) observed AGP label in the shank of *Nicotiana tabacum* pollen tubes, associated within the callose layer. While the localization of AGPs within this inner layer is a matter of debate (Taylor and Hepler 1997), label in the pollen tube shank was also found in *Nicotiana alata* (Ferguson et al. 1999) and *Lilium longiflorum*. Only in the latter was label also observed in the pollen tube tip (Jauh and Lord 1996). AGPs and other hydroxyproline-rich glycoproteins have also been identified in the extracellular matrix of the stigmatic papillae and stylar transmitting tract cells. Therefore various functions for this group of proteins in the fertilization process such as pollen tube-pistil adhesion, pollen tube nutrition, pollen tube guidance and recognition have been proposed (Cheung and Wu 1999; Jauh and Lord 1996; Sommer-Knudsen et al. 1997; Wu et al. 2001).

As is the case for angiosperms, gymnosperm pollen tubes appear to be rich in cell wall AGPs (Mogami et al. 1999). In some species they were even purported to represent the main cell wall component, as pectins and callose were said to occur in much lower amounts. These statements are based on

the comparison of fluorescence intensity after label with different antibodies, however, and need to be corroborated by more data (Mogami et al. 1999).

AGPs have also been suggested to be implicated directly in the tip growth process of pollen tubes, since $(\beta$-D-Glc$)_3$ Yariv agent, which binds to AGPs, significantly reduced pollen tube growth and led to abnormal expansion of the tube tip in some but not all species (Jauh and Lord 1996; Lord et al. 1996). In *Lilium* the agent seemed to destabilize the normal intercalation of new cell wall subunits, while exocytosis still occurs (Roy et al. 1998). This disrupts wall assembly at the tip, leading to segregation of homogalacturonans from a mixed callose/AGP component. Other studies using the JIM13 antibody to localize AGPs showed that the lily results do not apply to all species (Mollet et al. 2002). Further work with the Yariv reagent showed that affected tubes could regenerate new tips, that were strongly labelled by JIM13, leaving a labelled collar at the site of new tip emergence (Mollet et al. 2002).

The family of hydroxyproline-rich proteins that is best characterized are the Pex (Pollen extensin-like) proteins. They are specifically expressed in pollen grains and pollen tubes and have been characterized in maize and tomato pollen; homologs have been identified in other species. They are glycoproteins with a C-terminal extensin-like domain and an N-terminal globular domain that contains a leucine-rich repeat (LRRs) region adjacent to a hypervariable, possibly species-specific region (Bedinger et al. 2001; Rubinstein et al. 1995; Stratford et al. 2001). Pex proteins are very tightly associated with the pollen tube wall and are localized in the inner callosic sheath. The role of these proteins is still unclear. They have been suggested to have a structural function, but the LRR motif indicates that they might also be responsible for ligand binding and thus possibly for pollen-pistil recognition.

In addition to proteins that have a direct or indirect structural function, the pollen tube cell wall also interfaces with integral plasma membrane proteins that transduce signals from the extracellular to the intracellular space. Very few of these proteins have been characterized so far. Among them are pollen-specific receptor kinases which are likely to be involved in perceiving extracellular cues during pollen tube growth as they seem to span the entire cell wall (Kim et al. 2002; Muschietti et al. 1998). Other receptor kinases are involved in self-incompatibility (de Graaf et al., this volume).

Another family of proteins that is likely to be of importance for pollen tube growth are the wall associated kinases (WAKs). WAKs have a cytoplasmic serine/threonine protein kinase domain, span the plasma membrane and extend a domain into the cell wall (He et al. 1999). Similar to some AGPs, they physically link the plasma membrane to the carbohydrate matrix as they are covalently linked to pectin. Contrary to AGPs they are, however, able to directly signal cellular events through their kinase domain (Kohorn 2001). Their potential involvement in pollen tube growth is therefore rather likely, but needs to be shown experimentally.

3
Cell Wall Synthesis

3.1
Synthesis of Pollen Tube Walls

Information on the synthesis of somatic cell walls (Fry 2004) may not be immediately transferable to the study of pollen tube wall synthesis. The two wall layers found in pollen tubes (Sect. 2 above) are made by two different wall-synthesising systems. These are the integral membrane protein complexes on the plasma membrane and the endoplasmic reticulum and Golgi enzyme systems, whose products are packaged into secretory vesicles for transport to the tube tip and side walls.

The rate of cell wall synthesis is not closely correlated with the rate of tube extension. Hence faster growing tubes have thinner walls than slower growing counterparts (Steer and Picton 1984). Tubes transferred from favourable to less favourable conditions immediately form thicker walls at their tips (VanAelst and VanWent 1992) and switching between these two growth conditions results in a series of thickenings or rings in the tube wall (Li et al. 1996).

3.2
Cell Wall Components Delivered by Secretion

Synthesis of non-cellulosic wall polymers in somatic cells has been partly characterized, with the identification of end products, repeating units and the tranferase enzymes involved in assembly (Fry 2004). In pollen tubes the situation is much less clear. Xyloglucans, a major component of somatic cell walls, appear to be absent, or only present in small amounts (see Sect. 2 above) which is consistent with the low level of cellulose present. The extensive investigations into wall synthesis by the Golgi of somatic cells have not been replicated in pollen tubes. Microscopy reveals a high level of Golgi bodies in the cytoplasm of vegetative cells during pollen grain formation. These are either activated before pollen release (in trinucleates), forming a significant store of secretory vesicles in the pollen grains, or their activation is delayed until germination, so that initial tube growth rates are slow (lily and some other binucleates).

Transport of vesicles from the Golgi bodies to the tip is dependent on the actin microfilament network (Yokota and Shimmen, this volume). Vesicles fuse with the plasma membrane at the tip regardless of the rate of tip extension. Inhibition of this process leads to the accumulation of vesicles behind the tip, and at a rate which verified the estimates for the formation of vesicles (Picton and Steer 1981, 1985). Dissociation of vesicle fusion rates from tip growth rates can be demonstrated by transferring in vitro grown pollen tubes from optimal to sub-

optimal media, leading to a reduction in growth rate (Picton and Steer 1983; Roy et al. 1999). No accumulation of vesicles in the tip region is observed and these slower growing tubes have thicker walls at the tip than their faster growing counterparts, so wall thickness is an inverse correlate of vesicle delivery rates and tip mechanism extension rates (Steer and Picton 1984). Under sub-optimal growth conditions, vesicles deliver more membrane to the tip than is required for tube extension (Picton and Steer 1983) so the reasonable assumption has been made that endocytotic mechanisms retrieve the excess membrane (for detailed review see Malhó et al. 2005).

The main pectin components secreted by vesicles at the tip are methylesterified pectins (Geitmann et al. 1995; Li et al. 1994; Li et al. 1996). After their release at the tube tip, they are attacked by methyl esterases, which replace the neutral methyl groups with acidic carboxyl groups. These ionize to form negatively charged groups which readily bind divalent calcium ions that bridge adjacent pectin molecules together stabilizing the new wall (Holdaway-Clarke et al. 1997). The progression of de-esterification from the tip is easily demonstrated using JIM antibodies (Li et al. 1994). JIM7 labels the tip region and Golgi vesicles, indicating the presence of esterified pectin, whereas JIM5 labels the outer layer of the tube wall, not the tip, indicating de-esterified pectin. This sequence probably differs little from that occurring in somatic cells.

3.3
Cell Wall Components Synthesized by Plasmalemma-localized Enzymes

Pollen tubes have integral plasma membrane protein complexes that synthesise callose and possibly cellulose. Callose is synthesised in a number of somatic tissues, mainly around phloem sieve tube plates. In wounded cells it is proposed that cellulose synthases switch to callose synthesis in response to a loss of cell turgor and/or a depolarization of the plasma membrane. A surprising result of the characterization of the callose synthase enzyme from tobacco pollen cultured in vitro was that it is very different from that found in somatic plant tissues (Schlüpmann et al. 1993); it lacks a requirement for calcium ions, but is activated by a mild digestion with trypsin. The pollen tube callose synthase is located in the plasma membrane (Turner et al. 1998), but appears to originate from inactive precursors in the cytoplasm (Li et al. 1999) that are first incorporated into the plasma membrane and then activated. Callose synthase activity is first detectable 30 μm behind the tip (Ferguson et al. 1999) but the reason for this delay is not clear. A possible explanation is that it takes time to install and activate the enzyme. Typically in vitro pollen tubes extend at about 5 to 30 μm per minute, so only a few minutes elapse between insertion and activation. Interestingly, not all the synthase is activated on incorporation into the plasma membrane, since it is always possible to increase the level of callose synthase activity of plasma membrane isolates by incubat-

ing them with trypsin (Li et al. 1999). This latent enzyme has been postulated to be activated later at sites of callose plug formation (Li et al. 1999).

A callose synthase gene, *NaGsl1*, is expressed abundantly in pollen tubes of *Nicotiana alata* (Doblin et al. 2001). This gene is not expressed in other tissues, and so it is probably providing transcripts for the unique callose synthase found in these tubes. Levels of *NaGsl1* transcripts were high in mature pollen, suggesting that pollen grains are released with the appropriate transcripts for wall formation in place before germination. Transcript levels remained high during tube growth (Doblin et al. 2001).

There are claims that cellulose synthesis in pollen tubes occurs earlier than callose synthesis, as cellulose can be detected 5–15 μm behind the tip (Ferguson et al. 1999). However there is still some doubt about the presence of true β-1,4 cellulose fibrils in pollen walls. Earlier claims that cellulose synthesizing rosettes are visible in freeze etched preparations of pollen tubes have yet to be verified (Reiss et al. 1985). Cellulose specific antibodies do label tubes down to the tip, though there are problems of antibody specificity and of producing a section in the median longitudinal plane of a tube that cast doubt on such claims. Applying 2,6-dichlorobenzonitrile, an inhibitor of cellulose synthesis, to in vitro growing pollen tubes of *Lilium* and *Petunia* led to the disruption of tube growth with distorted and bulbous tubes being formed, and some tubes burst (Anderson et al. 2002). This was interpreted as evidence that cellulose is required for normal growth. Similar results were seen when conifer pollen was grown in the presence of isoxaben, another inhibitor of cellulose synthesis (Lazzaro et al. 2003).

Genetic approaches to the study of cellulose synthesis have been introduced in vegetative plant cell studies (Richmond 2000). These show that one set of *CesA* genes is turned on for synthesising primary walls and a different set for secondary walls (Taylor et al. 2003). Analysis of the expression of cellulose synthase genes in pollen tubes of *Nicotiana alata* has revealed that a cellulose-synthase-like gene, *NaCslD1*, is expressed abundantly in pollen tubes, but not in other tissues, except roots, where it could be involved in root hair formation, and in moss rhizoids (Doblin et al. 2001). The true cellulose synthesis gene, *NaCesA*, found in somatic cells, could not be detected in the pollen tube cDNA library. Although no definitive evidence exists for the function of *Csl* genes, Doblin et al. (2001) conclude that the *NaCslD1* gene is responsible for cellulose synthesis in these pollen tubes.

Growth of the pollen tube wall could conceivably involve modification of components after their insertion into the wall structure, as in somatic cell wall growth. β-glucanases capable of degrading both 1,3- and 1,4-linked polymers have been isolated from in vitro grown lily pollen (Kotake et al. 2000). Their relevance to tube growth was tested by growing tubes in the presence of glucanase inhibitors which do affect somatic cell growth. These inhibitors reversibly suppressed tube growth, with increases in tube diameter (Kotake et al. 2000). Renewed growth, as so often happens in pollen tubes, occurred laterally

to the inhibited apex. One of these glucanases is highly specific for pollen grains and tubes, but another was found in all the lily tissues tested (Takeda et al. 2004). It was proposed that glucanase-mediated hydrolysis of callose and a 1, 3 : 1, 4-β glucan is involved in the regulation of tube elongation.

4
Cell Wall as Ion Storing and Transit Compartment

The structure of the cell wall has a profound effect on its chemical and permeability properties, both of which will impinge on the physiology of the enclosed protoplast. Calcium ions have been known to affect growth of in vitro pollen tubes (Malhó, this volume and Hepler et al., this volume). As discussed above, esterase activity leads to generation of pectic carboxylic acids that bind calcium ions (Carpita and Gibeaut 1993). At the endogenous levels of apoplastic pH and calcium ions in pistil tissues, or in growth media, these acidic sites become saturated with calcium soon after they are formed. The calculated demand for calcium entry into this growing wall was suggested to match the observed flux rate of calcium into the tube tips (Holdaway-Clarke et al. 1997).

Wall permeability is thought to be largely controlled by callose. The pioneering work of Jaffe on ionic currents around pollen tubes defined the sites and fluxes of ions and protons (Jaffe et al. 1975), and this has been extended by a number of other authors (see Hepler et al., this volume and Sze et al., this volume). Cations such as protons, calcium and potassium enter at the tip which is free of callose, and is covered by neutral esterified pectins. The conclusion from these studies is that mature pollen tube walls are relatively impermeable to ions when compared to the apical wall.

The positioning of calcium ion influx on the apical dome determines the direction of tube growth (Malhó and Trewavas 1996), and tubes respond to changes in the direction of the external calcium gradient by bending towards the ion source. This suggests that the callose sheath might contribute to restricting calcium ion entry to the tip region where the signal can be detected and growth direction altered through a shift in the point of secretion.

5
Cell Wall as Architectural Component – Mechanical Properties

In general, the process of plant cell growth is driven by the controlled yielding of the existing cell wall under an applied pressure, while simultaneously new cell wall material is inserted. While the mechanism of tip growth is often considered fundamentally different from that of diffuse growth, this difference is perhaps quantitative rather than qualitative. Similarly to diffuse growth, turgor is believed to be the primary motive force behind tip growth even though

growth rates cannot directly be correlated with the amount of turgor present (Benkert et al. 1997). This, however, does not contradict the notion of turgor as main propulsive force since internal pressure might simply be a necessary *conditio sine qua non* whereas other features determine the rate of elongation. Among these might be an adjustable yield threshold and strain hardening capacity of the apical cell wall, and – at least providing an upper limit for elongation rate – the rate of insertion of new cell wall material.

Green (1969) and others have proposed that tip localized expansion must be caused by tip to base changes in the physical properties of the wall since the turgor force is non-vectorial. In other words, the cell wall in the shank of the tube needs to be more resistant to tensile stress than the apical cell wall to assure that the latter yields first. However, a slight difference in stress resistance is not sufficient; for geometrical reasons this difference actually has to be bigger than a factor 2. The physical laws for thin-walled pressure vessels state that in a cylindrical structure the tensile wall stress in circumferential direction is twice as high as the tensile stress in longitudinal direction (Fig. 7). The tensile stress in the wall of the very apex of this cylindrical structure is identical to the one present in longitudinal direction in the shank. Given a uniform, isotropic wall, a cylindrical pressure vessel should, therefore, balloon to become spherical or burst producing a longitudinal slit in the shank if pressure rises above a certain threshold. This is obviously not the case in pollen tubes even though they are highly pressurized. Therefore, in order for the cell wall at the tip region to yield first, it needs to start yielding at a pressure that is lower than half the pressure

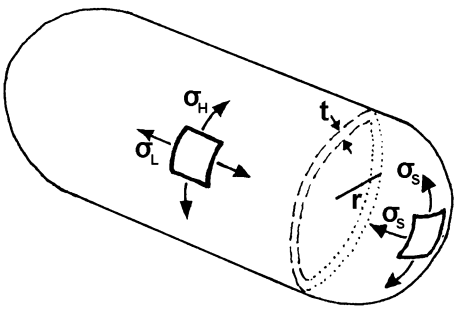

Fig. 7 Distribution of tensile stress in the wall of a thin-walled cylindrical pressure vessel. The stress around the wall must have a net resultant to balance the internal pressure across the cross-section. In the spherical apex and along the longitudinal direction in the shank of the cylinder this means: $\sigma \cdot t \cdot 2\pi r = p \cdot \pi r^2$ with σ – stress, t – thickness of the wall, r – radius of the cylinder or sphere, p – internal pressure. From this equation follows that $\sigma = pr/2t$, which applies to both σ_L – the longitudinal stress in the cylindrical part of the vessel and σ_S – the stress in the spherical apex. For the stress in circumferential direction in the cylinder the following equation applies: $2 \cdot \sigma \cdot t \cdot dl = p \cdot 2 \cdot r \cdot dl$ with dl – length of the cylinder. Therefore the stress in the wall in circumferential (hoop) direction is $\sigma_H = pr/t$. Consequently, the stress in hoop direction in the cylinder is twice as high as the stress in the apex: $\sigma_H = 2 \cdot \sigma_S$

necessary to produce the shank located ballooning or bursting. This requires a considerable difference in the yield threshold between the two regions. While the physical necessity for this gradient is compelling, experimental evidence for its presence was produced only recently. Micro-indentation demonstrated that the local cellular stiffness in growing pollen tubes is considerably lower at the apex compared to the shank of the tube (Fig. 8) (Geitmann and Parre 2004). Several cell wall components might be responsible for the creation of this gradient since they are either deposited in non-uniform manner or show a non-uniform distribution in their chemical configuration. The most prominent structure that is absent at the tip, but present in the shank is callose. Micro-indentation of pollen tubes in the presence of a callose digesting enzyme revealed that in the shank, callose contributes to resistance of the tubes against compression forces. The same enzyme induced an increase in cellular diameter thus suggesting that this cell wall component is also involved in the tensile resistance of the cell wall against circumferential stress caused by the turgor pressure (Parre and Geitmann 2005a).

Cellulose seems to be of lower importance in the physical cell wall properties determining tip growth. While microfibrils seem to be confined to the shank in some species (Ferguson et al. 1998), they are present in the apex in others, particularly in gymnosperm pollen tubes (Derksen et al. 1999; Lazzaro et al. 2003). No clear picture has emerged, especially since knowledge about the orientation of microfibrils in pollen tubes is scarce. An early study had indicated that microfibril orientation is perpendicular to the long axis (O'Kelley and Carr 1954), which would allow them to contribute to the

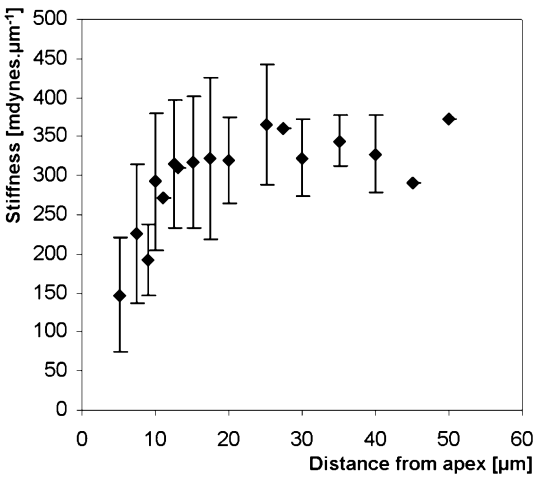

Fig. 8 Graph plotting stiffness versus distance from the apex of a typical growing *Papaver rhoeas* pollen tube. Measurements were done with the micro-indenter. In the apical 20 µm a steep increase of stiffness can be noted whereas no significant variations occur in the distal area. (Figure reproduced with permission from Geitmann and Parre 2004)

cell wall's resistance against tensile stress caused by turgor pressure. Sassen (1964), on the other hand, reports helicoidal orientation of microfibrils in the shank which would theoretically allow for expansion, albeit limited, of the cylindrical part of the tube in both radial or longitudinal direction.

The cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile, caused tube distortion or rupture in petunia and lily pollen, accompanied by a change in cell wall organization (Anderson et al. 2002), suggesting that cellulose is required for normal tube growth. In the *Petunia* pollen tubes rupture occurred at a distance of 17 μm from the tip thus indicating that neither the very tip of the pollen tube nor the distal part of the shank depend (solely) on reinforcement by cellulose in this species. The subapical region lacks a significant layer of callose and has a pectic layer that is prone to bursting.

Picea abies pollen tubes, on the other hand, which are rich in cellulose at the apex showed swelling upon application of isoxaben, an inhibitor of cellulose synthesis (Lazzaro et al. 2003). While this was partly attributed to a disorganization of microtubules through communication across the plasma membrane between cortical microtubules and cellulose microfibrils, it also indicates that in this species, cellulose contributes to the mechanical stability of the pollen tube tip.

The pectin layer has also an important role in the mechanical properties of the wall. Apex-located pectins are almost always of the methyl-esterified type whereas the shank pectins are acidic or scarcely esterified. This is an essential feature as de-esterification allows pectins to become gelated in the presence of calcium ions. This gelation process considerable increases the Young's modulus of this matrix component (Jarvis 1984), thus potentially increasing resistance against tensile stress. The first indication that this principle holds true for in vitro pollen tube cell walls has been demonstrated only recently by micro-indentation. Parre and Geitmann (2005b) demonstrated that de-esterification of the apical cell wall using pectin methyl esterase considerably increases the local stiffness of the cell in this region, presumably by increasing cross-linking by calcium, and is able to prevent pollen tube elongation (Bosch et al. 2005; Parre and Geitmann 2005b). A delicate control over the gradient of esterification is thus essential to prevent mechanical instabilities. Regions further back from the apex are stabilized by other cell wall components (most likely callose) as demonstrated by the partial digestion of pollen tube pectin which causes the swelling of only the apical 30 μm of the tube (Parre, Geitmann, unpublished). However, the cylindrical region between the apex and the 30 μm mark seems highly dependent on the presence of pectin and the resistance against circumferential tension stress provided by the increasing pectin gelation in this region. The importance of the pollen's own pectin methylesterase for this process was demonstrated by the characterization of VANGUARD1, a pectin methylesterase expressed in *Arabidopsis* pollen tubes. Functional interruption of the gene in *vdg1* mutants causes pollen tubes to be very unstable and show a high frequency of bursting (Jiang et al. 2005). This is consistent with results

of the expression of a flax pectin methylesterase antisense sequence in *Nicotiana tabacum*, which reduced pollen germination, even though effects on cell wall stability were unfortunately not provided (Lacoux et al. 2003). Obviously, demethylation by itself is not enough to ensure a change of physical properties of the pectin polymers. Calcium ions actually have to be present to allow their gelation. Experiments in which the calcium content of the surrounding medium was lowered revealed that the pollen tube lost its ability to resist tension and burst. Interestingly, these bursting events occurred close to but not at the very apex, indicating that this flanking region is indeed a critical one, the stability of which depends on the process of de-methylesterification and calcium gelation (Shen and Geitmann, unpublished).

The cell wall at the apex has to cope with a highly sensible equilibrium since it has to yield to the pressure but not burst. Changes in the osmotic pressure of the surrounding medium lead to bursting, if lowered, or failure of elongation, if raised. In addition, new cell wall material, delivered continuously by vesicles, has to amalgamate with the existing cell wall that is simultaneously being deformed. Failure of vesicle fusion in flavonol-deficient *Petunia* leads to bursting of pollen tubes (Derksen et al. 1999). Delicately balanced forces, therefore, govern this highly dynamic apical growth process. These are largely unknown and warrant further research.

Another poorly understood topic is how the growing pollen tube is able to exert penetrating forces. Invasion of the transmitting tissue is most certainly facilitated by the secretion of cell wall digesting enzymes or expansins causing a separation of transmitting tract cells (Cosgrove 1997; Heslop-Harrison 1977; Hiscock et al. 1994). Nevertheless, it is reasonable to presume that the pollen tube still has to exert some penetrating force to pave its way to the ovary. The increased external mechanical resistance provided by the transmitting tissue might contribute to the equilibrium between pollen tube cell wall and turgor, but it is unknown, whether turgor in in vivo growing pollen tubes is higher than in their counterparts in vitro, or whether perhaps the yielding threshold of the cell wall is altered to allow the turgor to act against mechanical obstacles. A first indication is provided by the fact that pollen tubes grown in agarose – an artificial mechanical obstacle – show reduced label for callose compared to liquid grown pollen tubes (Parre and Geitmann 2005a). However, studies comparing the in vivo with the in vitro situation – both concerning cell wall composition and growth behaviour will be needed to answer these questions.

6
Adhesion and Interaction with the Transmitting Tissue

In addition to being a mechanical structure, the pollen tube cell wall forms the interface between the cell and its surroundings. This interface represents

a very intimate contact between two different organisms – the male gametophyte and the female sporophyte. Signals that are exchanged between these two organisms need to pass through the cell wall to reach the respective receptor on the plasma membrane or in the cytoplasm. The cell wall, therefore, is an important arena for complex interactions such as those occurring during self-incompatibility reactions (de Graaf et al., this volume).

To allow the passage of signalling and other molecules, the cell wall needs to have a certain porosity. The permeability of *Gladiolus* pollen tubes grown in vitro was restricted to globular molecules with a molecular weight from 15 to 25 kDa (Hoggart and Clarke 1984), whereas *Nicotiana* tubes had a cut off at about 10 kDa (O'Driscoll et al. 1993). Analyses done on the class III pistil-specific extensin-like proteins (PELPIII) in Solanaceae, on the other hand, have shown that these glycoproteins are translocated into the callosic wall of the pollen tube albeit having a molecular weight of 110–140 kDa, thus suggesting that the cell wall has a rather high degree of permeability (de Graaf et al. 2004). Whereas the outer pectic layer is rather permeable, it remains to be confirmed if the callosic layer has some controlling function regarding penetration of molecules.

The interaction between pollen tube and style has also important mechanical aspects. In solid-styled species the tubes are likely to be guided by the longitudinal configuration of the cells composing the transmitting tissue. Intercellular growth parallel to the longitudinal axis of the style is thus the mechanically easiest path. During this stylar phase of growth, pollen tubes might perhaps not even need any chemical cues before they reach the ovary, where guidance becomes a more complex problem. In hollow styles on the other hand, pollen tube growth is less confined spatially and probably needs additional cues. In lily, a hollow styled species, adhesion of pollen tubes to the transmitting tract epidermis has been shown to be based on a pectic polysaccharide and a stigma/stylar cysteine rich adhesin (SCA) present in the stylar exudate. Together these two molecules are bound to the surface of the transmitting tract cells and cause adhesion of the pollen tubes to this surface and to one another (Lord 2003). The receptor in the pollen tube is however unknown (Johnson and Lord, this volume).

7
Generative and Sperm Cell Walls

The male gametophyte is formed by the first pollen grain mitosis. The division is asymmetric and the small generative cell so formed migrates into the cytoplasm of the vegetative cell, separated from it by the plasma membranes of each cell and the thin layer of pectic wall material (Raghavan 1997). In some species, a temporary callose wall is deposited in this thin pectic layer soon after the pollen grain mitosis. This has been confirmed by aniline blue

and resorcinol blue staining (see references in Raghavan 1997). This is reminiscent of the callose walls seen at pollen mother cell meiosis, and may be a further protection against infection of the germ line by viruses.

The existence of plasmodesmata in the generative cell wall is disputed (Cresti et al. 1987; Tiwari 1994) but thin sections of chemically-fixed material do show occasional contacts between the plasma membranes of the vegetative and generative cells. To call these plasmodesmata would require the demonstration of endoplasmic reticulum continuity between the two cells via a desmotubule within the tube of plasma membrane. To date no such images have been published. In contrast transfer cell-like wall ingrowths have been recorded. These are indicators of apoplastic transfer of nutrients to the inner cell (Tiwari 1994). In some pollen grains the generative cell is deeply lobed, which possibly serves the same purpose (Noguchi and Ueda 1990; Southworth et al. 1994).

While AGPs were shown to be present in both membranes surrounding the generative cell (Mogami et al. 1999; Southworth and Kwiatkowski 1996), there is no information on the molecular structure of the carbohydrates in this generative cell wall, and certainly no mechanically strong macromolecular organization is required, as the generative cell exists in isotonic equilibrium with the cytoplasm of the vegetative cell. In binucleate pollen grains, the generative cell must assume a spindle shape so that it can progress into and down the pollen tube before mitosis to form the sperm cells. This change in shape is achieved by the formation of longitudinal bundles of microtubules (Burgess 1970). Such a change is unique in higher plants, where cell shaping is usually driven by osmotic turgor pressure inflating a mechanically asymmetric cell wall. In generative cells it is the internal cytoskeleton that exerts shape control, as in many animal cells (Cai and Cresti, this volume).

Generative cell mitosis yields two sperm cells that lie in the same cavity within the vegetative cell. These sperm cells have even more tenuous cell walls than the generative cell. Their walls, and more specifically the outer faces of their plasma membranes, are of great importance. This is because the sperm cells are not identical, in fact only one is a true sperm cell able to initiate fertilization and zygote formation. In some species the sperm are dimorphic, so the true sperm cell can be identified morphologically. It is assumed that there must be some subtle differentiation in isomorphic sperm cells, so that one will recognize the egg cell plasma membrane and the other the central cell plasma membrane. Intensive research has been directed to identifying the recognition molecules involved. Antibodies have been raised against purified fractions of sperm cell plasma membranes. The glycoproteins detected differ from those found on control somatic cells and contain mannosyl and glucosyl residues (Xu and Tsao 1997). Furthermore, the lectin binding sites of generative cells are reorganized and modified during formation of the sperm cells (Fang and Sun 2005), possibly in preparation for fertilization events. However, it has not been possible to distinguish between the two sperm cells using these methods.

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Cellular Mechanisms for Pollen Tube Growth Inhibition in Gametophytic Self-incompatibility

Barend H. J. de Graaf[1] · Chris Lee[2,3] · Bruce A. McClure[3] · Noni (V. E.) Franklin-Tong[1] (✉)

[1]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
v.e.franklin-tong@bham.ac.uk

[2]Division of Biological Sciences, University of Missouri–Columbia, 105 Tucker Hall, Columbia, MO 65211-7310, USA

[3]Department of Biochemistry, University of Missouri–Columbia, 105 Life Sciences Center, Columbia, MO 65211-7310, USA

Abstract Self-incompatibility (SI) is a mechanism used by angiosperms to prevent self-fertilization. Here we review current knowledge of two different gametophytic SI systems at the cellular level, revealing different mechanisms that interfere with pollen tube growth. In the *Solanaceae*, *Rosaceae*, and *Scrophulariaceae*, SI is controlled by an interaction between a pistil component, S-RNase, and a pollen component, an F-box protein, SLF/SFB. While a variety of models focused on ubiquitylation have been explored, it is still unclear exactly how the S-RNase based system operates at the cellular level. In *Papaver*, entirely different S-proteins act as signalling ligands that trigger a Ca^{2+}-dependent signalling cascade that results in programmed cell death (PCD). Although the pollen *S*-receptor has not been identified in *Papaver*, the mechanisms involved in inhibiting incompatible pollen are better understood.

1 Introduction

Angiosperms display an enormous diversity of flower morphology and reproductive mechanisms. Successful sexual reproduction, however, is dependent on the accomplishment of the same series of contingent events: delivery of pollen to a stigma, pollen hydration, germination, growth through the pistil to the ovary, and, finally, fertilization. As pollen grows, it is in contact with a pistil-secreted extracellular matrix (ECM), comprising various components that interact with pollen (see Sze et al., this volume, and Johnson et al., this volume). Proteins required for the recognition and rejection of self-pollen are secreted into the ECM while other proteins that don't have any role in controlling pollination (e.g., proteinase inhibitors), may help protect the pistil from pathogen invasion (Atkinson et al. 1993). Essentially, the ECM acts as the "staging ground" for pollen-pistil interactions and creates an optimum structural and physiological environment for pollen germination and tube growth. Thus, pollen-pistil interactions involve many proteins that serve a variety of

functions, including nutrition, signal recognition, signal transduction, and precise temporal and spatial regulation of polar tube growth (see Sze et al., this volume).

The pollen tube elongates through the pistil ECM by tip growth, which requires precise delivery and fusion of secretory vesicles, delivering new pollen tube plasma membrane, membrane proteins and cell wall precursors to the tube apex (de Graaf et al. 2005). Directional growth requires that signals from the pistil are received, transduced, and interpreted by the pollen's physiological apparatus. Cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) at the tube tip, a functional actin cytoskeleton, and small GTPases (Rops and Rabs) are all required for this regulated directional polar growth (see Malhó, this volume; see Hepler et al., this volume; and see Yokota et al., this volume).

Control mechanisms operate to restrict mating both between and within species. Many angiosperms possess SI systems to avoid inbreeding and to promote outcrossing. SI is a cell–cell recognition system that discriminates against genetically related pollen (i.e., "self" or incompatible pollen); incompatible pollen is rejected by the pistil whereas compatible pollen is allowed to grow. In SI systems, the timing of pollen rejection varies enormously between species. It may take place during pollen hydration on the stigma (e.g., in *Brassica*), during pollen germination (e.g., in *Papaver rhoeas*), during pollen tube growth through the style (e.g., in *Solanaceae*), or while in the ovary either prior to fertilization or through "abortion" (Sage et al. 1994). The mechanisms controlling SI are complex. In recent years, significant progress has been made in identifying some of the molecular components involved. Here, we review some of these advances in the field of SI and place them in a cellular context.

2
Basic Principles of Self-incompatibility (SI)

In most species, SI is genetically controlled by the *S*-locus, which is highly polymorphic and multi-allelic. The genes involved in SI specificity have three general requirements: 1) they must be linked to the *S*-locus; 2) they must be sufficiently polymorphic to specify the large number of alleles at this locus; and, 3) they must have the correct tissue-specific and developmental expression patterns. At least two genes are involved – one expressed in the pistil (pistil-*S*) and one expressed in the pollen (pollen-*S*). By definition, *S*-specific pollen rejection results from the interaction of pollen-*S* and pistil *S*-specificity determinants. However, it is clear that in some SI systems, other non-*S*-locus factors are also required. The basic principle of SI is that pollen carrying a particular *S*-haplotype can only fertilize plants carrying different *S*-haplotypes. Whenever pollen lands on the stigma of a plant with the same *S*-haplotype, the SI mechanism prevents pollen from achieving fertilization at some stage of pollination.

Although SI was considered to be an ancestral angiosperm trait, analysis of *S*-gene sequences revealed that SI arose independently in several lineages (Matton et al. 1994). Two types of SI mechanisms have been described for homomorphic plants: sporophytic SI (SSI) and gametophytic SI (GSI). SSI is rare; it has been found in *Brassicaceae*, *Asteraceae*, and *Convolvulaceae*. GSI is widespread, occurring in the *Solanaceae*, *Papaveraceae*, *Ranunculaceae*, *Leguminosae*, *Onagraceae*, *Scrophulariaceae*, *Rosaceae*, and *Poaceae* (Brewbaker 1957; Igic and Kohn 2001). In GSI, incompatibility is determined by the *S*-haplotype of the haploid pollen. If the pollen's *S*-haplotype matches either of the two *S*-haplotypes in the pistil, then *S*-specific inhibition of that pollen occurs. If the pollen *S*-alleles are different, then the pollen is considered compatible and is not rejected. Studies on SSI will not be discussed further. For a recent review on SSI, see Takayama and Isogai (2005).

3
Two Contrasting GSI Systems

Two different GSI systems have been extensively studied. SI species in the *Solanaceae*, *Rosaceae*, and *Scrophulariaceae* display S-RNase-based GSI systems. In contrast, *Papaver rhoeas* (field poppy) is controlled by completely different pistil S-proteins. There are critical differences between these two GSI systems. The stigma of *Papaver* is "dry", while that of the *Solanaceae* is "wet". The timing and location of pollen rejection also differ, though in both systems incompatible pollen tubes are inhibited as they grow through secretions containing the pistil *S*-determinants. In *Papaver*, pollen rejection occurs within minutes during germination on the stigma surface. In the S-RNase based system, pollen rejection can take hours and occurs as the pollen tube grows through the ECM of the style transmitting tissue (TT).

Both the pollen-*S* and pistil-*S* have been identified in the S-RNase based system. In the pistil, *S*-specificity is determined by S-RNases, which are an abundant component of the pistil ECM. In the pollen, *S*-specificity is determined by SLF/SFB, an F-box protein. In *Papaver*, the pistil-*S*-specificity determinant is S-protein, a small secreted signalling ligand. The pollen *S*-component has not been identified, but it is assumed to be a receptor.

4
Cellular Mechanisms of GSI in Solanaceae

Morphological changes in incompatible pollen tubes may provide clues about the SI mechanisms. Pollen tube growth inhibition in the S-RNase-based system is characteristically slow. Electron microscopy studies (Geitmann et al. 1995) revealed that compatible and incompatible pollen tubes cannot be dis-

tinguished during the first few hours after pollination. After eight hours, incompatible pollen tubes have a concentric endoplasmic reticulum (ER), the fusion of secretory vesicles (SV) decreases resulting in an accumulation of vesicles in the tube apex, and thickening of the inner callosic and outer pectic pollen tube wall are detected. Later, the cytoplasm of incompatible pollen tubes degenerates. It is not known how these morphological changes arise, or how they relate to the physiology of pollen tube growth. Ultrastructural changes observed in incompatible pollen tubes are similar to necrosis or programmed cell death (PCD). Necrosis is a passive process triggered by cytotoxic activity, characterized by swelling and disruption of internal compartments. PCD, on the other hand, is an active process. Based on the evidence available, S-RNase-based GSI probably involves necrosis (Geitmann, 1999), but additional research is needed to resolve whether changes in the endomembrane system are a cause or a consequence of GSI mechanisms.

4.1
S-RNase Determines S-specificity in the Pistil

The candidates for pistil-encoded *S*-glycoproteins in *Nicotiana alata* were identified by pistil protein segregation analysis. Anderson et al. (1986) cloned the first pistil-*S* cDNA that co-segregated with an *S*-genotype. They were named S-RNases because of their ribonuclease domains and activity (McClure et al. 1989). S-RNase expression both coincides developmentally with the onset of SI, and is restricted to cells forming the path from the stigma to the ovary. S-RNases contain secretion signals and are deposited in the ECM in high concentrations. Transgenic plant studies expressing cloned S-RNases provided definitive evidence that S-RNases are pistil-*S*. Expression of transgenic S-RNase allowed rejection of pollen containing the transgene's *S*-haplotype, demonstrating that S-RNase determines *S*-specificity (Lee et al. 1994; Murfett et al. 1994).

Sequence analysis of *S-RNase* genes showed high levels of polymorphism. They contain highly divergent hypervariable (HV) regions (Ioerger et al. 1991). Site-specific mutagenesis experiments in *Solanum* showed that *S*-specificities can be changed by altering amino acids in the HV regions of S-RNases (Matton et al. 1997). Nevertheless, analyses of S-RNases in rosaceous and solanaceous species suggest that regions other than HV regions may be involved in recognition (Ishimizu et al. 1998). Structural analyses established that the most HV regions reside on the surface, where they could interact with pollen-*S* (Ida et al. 2001). However, how S-RNase specificity is determined remains unclear.

4.2 The Cytotoxic Model of S-RNase-based GSI

The association of RNase activity with GSI suggested that pollen tube growth is inhibited by a cytotoxic reaction. This hypothesis was tested using ^{32}P-labeled pollen in both compatible and incompatible pollinations. Results showed that pollen RNA is degraded during incompatible pollen-pistil interactions (McClure et al. 1990). This observation formed the basis of the cytotoxic model of GSI in which self-S-RNases act as *S*-specific cytotoxins. Crucially, catalytically inactive mutants of *P. inflata* S_3-RNase cannot cause *S*-specific pollen rejection (Huang et al. 1994). This model (Fig. 1) assumes that pollen tubes take up S-RNases. Immunolocalization data later showed

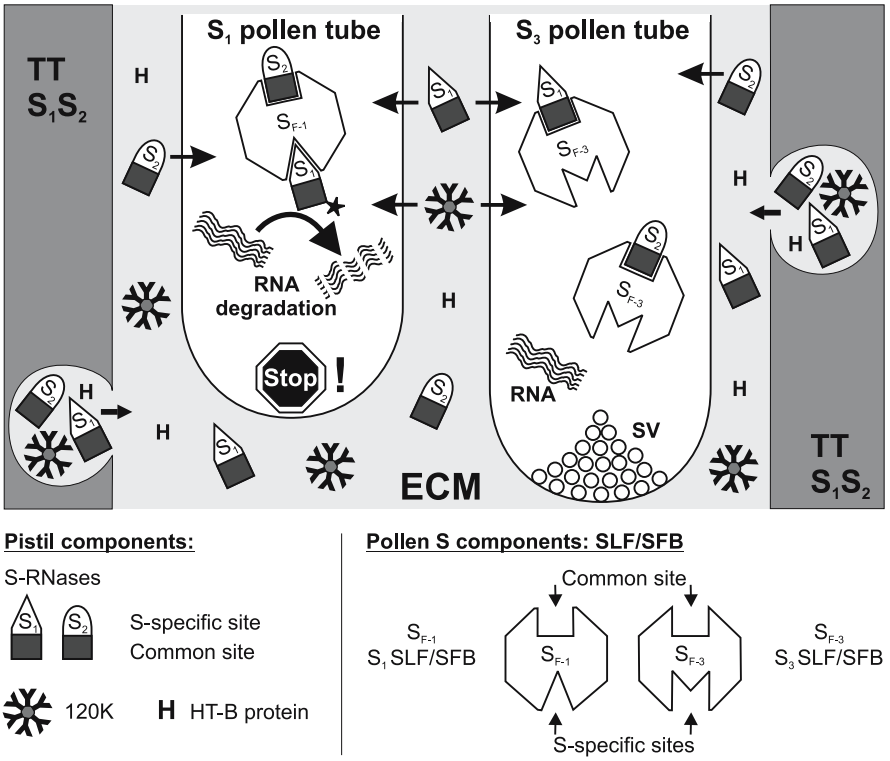

Fig. 1 Cytotoxic model of S-RNase-based SI. S-RNases, non-*S* factors (HT-B and 120K), and other components required for pollen tube growth regulation are secreted into the ECM by pistil cells. S-RNases contain a specific binding site and a non-specific binding site for the pollen *S*-component, SLF/SFB. In an incompatible situation (*left*), S-RNase causes degradation of pollen RNA leading to pollen rejection. In a compatible situation (*right*), non-specific binding of S-RNase to SLF/SFB inhibits its cytotoxic activity and pollen can grow

that S-RNases are present inside both incompatible and compatible pollen tubes, though they do not cause rejection in the latter (Luu et al. 2001). In incompatible pollinations, interaction between S-RNase and pollen-*S* (SLF/SFB) leads to degradation of RNA and inhibition of pollen tube growth. In a compatible pollination, RNA is not degraded, and pollen tube growth is normal. Pollen-*S* is thought to prevent S-RNase activity in compatible pollen tubes. Figure 1 shows two S-RNase binding sites on pollen-*S*. A common site is capable of binding any S-RNase, preventing S-RNase cytotoxicity. Binding to the *S*-specific site, on the other hand, leads to the cytotoxic reaction and to inhibition of pollen tube growth.

4.3
Non-S-RNase Factors are Required for S-specific Pollen Rejection

Genetic and molecular studies have shown that factors not linked to the *S*-locus are required for *S*-specific pollen rejection in S-RNase-based GSI. Two such factors have been identified at the molecular level: HT-B, a small asparagine-rich protein, and 120 K, a 120 kDa stylar glycoprotein (McClure et al. 1999). HT-B's pistil-specific expression coincides with the onset of SI. Antisense and RNAi experiments confirmed its requirement for *S*-specific pollen rejection in both *Nicotiana* and *Solanum* (O'Brien et al. 2002) and that HT-B does not affect S-RNase expression. Biochemical studies identified 120 K as a candidate for an SI factor (Cruz-Garcia et al. 2005) containing a highly glycosylated N-terminal domain, a conserved cysteine-rich C-terminal domain and size polymorphism between self-compatible (SC) and SI species. Moreover, it is taken up by pollen tubes in vivo (Lind et al. 1996). RNAi experiments demonstrated that 120 K has a specific role in *S*-specific pollen rejection; plants with suppressed 120 K lost *S*-specific pollen rejection without affecting S-RNase or HT-B expression. *4936-factor* is another non-S-RNase factor that has been linked to pollen rejection, but it has only been defined genetically (McClure et al. 2000). Plants that suppressed *4936-factor* lost *S*-specific pollen rejection without affecting S-RNase, HT-B, and 120K expression. The functions of these factors in pollen rejection are not known. Recent immunolocalization studies show that S-RNase uptake by the pollen tubes is not affected in *4936-factor* mutants, *HT-B* antisense plants, or 120 K RNAi plants, suggesting that these factors are not required for S-RNase uptake (Goldraij et al. 2006). Current speculation is that they interfere with some aspect of the pollen tube's physiology in order to inhibit growth.

4.4
Identification of Pollen-S as SLF/SFB

The *S*-specificity determinant in pollen is an ***S*-locus F-box** protein, named SLF or SFB (Kao and Tsukamoto, 2004). *SLF* genes were identified by sequenc-

ing genomic DNA adjacent to the *S-RNase* gene. *Antirrhinum* $AhSLF_2$ was the first *SLF* gene to be identified. It is located only 9 kb from S_2-*RNase* (Lai et al. 2002). *S-RNase*-linked *F-box* genes were identified independently in the *Rosaceae*; these were named *SFB* (***S***-locus **F**-**b**ox) (Ushijima et al. 2003). Sequence analyses of *SFB* genes provide evidence of positive selection, strongly supporting their role as recognition proteins (Ikeda et al. 2004).

Several key properties of SLF/SFB proteins are consistent with them being pollen-*S*. SLF genes are expressed in mature pollen and exhibit sequence polymorphism (Ushijima et al. 2003; Ikeda et al. 2004). A histidine-tagged $AhSLF_2$ protein and the yeast-two-hybrid system were used to demonstrate interaction between the C-terminal region of $AhSLF_2$ and S-RNase (Qiao et al. 2004), although binding was not *S*-specific. Together, these data support identification of SLF/SFB as the pollen *S*-determinant.

4.4.1 S-locus F-box Protein Determines *S*-specificity in Pollen

Transformation studies provided direct evidence that SLF/SFB determines *S*-specificity. In one study, $PiSLF_2$ was expressed in S_1S_1 and S_2S_3 *Petunia inflata*. This transformation was not expected to introduce new recognition specificity into pollen; rather, SI breakdown was predicted, because of the heteroallelic pollen (HAP) effect, which describes the loss of SI observed when two different *S*-haplotypes are expressed in pollen. This is exactly what was observed; *P. inflata* S_1S_1 plants expressing $PiSLF_2$ became SC. Similar results were observed when the *Antirrhinum* $AhSLF_2$ gene was expressed in self-incompatible *Petunia hybrida* S_3S_3 (Qiao et al. 2004). These experiments implicated *SLF* genes in SI, but did not directly demonstrate *S*-specificity. To address this issue, it had to be shown that a putative pollen-*S* gene affects *S*-haplotypes differently. An *S*-specific effect was demonstrated in *P. inflata* (Sijacic et al. 2004). Critically, $PiSLF_2$ did not cause breakdown of SI when it was expressed in conjunction with a native $PiSLF_2$ gene. S_2S_3 plants expressing $PiSLF_2$ were SC. However, only S_3-pollen expressing the transgene was compatible; S_2-pollen was rejected regardless of the transgene's presence.

To date, only the $PiSLF_2$ gene has been unequivocally shown to determine *S*-specificity. Given the multiplicity of these genes and the ambiguity of identifying orthologues across wide phylogenetic distances, a complete set of results in each family must be obtained to establish their roles. *S*-specificity experiments should be repeated in other species. Since multiple *S*-linked F-box genes are expressed in pollen, they could have other roles and should be tested for this.

4.4.2
What is the Function of F-box Proteins?

In plants, there are a large number of F-box genes (697 in *Arabidopsis thaliana* compared to 11 in yeast – Kipreos and Pagano 2000; Gagne et al. 2002), suggesting that they may serve a variety of roles. F-box proteins have been implicated in hormone-regulated gene expression, flowering, and photomorphogenesis (Gagne et al. 2002). At the biochemical level, they are best known for their role in ubiquitin-mediated protein degradation. Here, they act as substrate recognition factors for **S**kp1-**C**ullin-**F**-box (SCF) E3 ubiquitin ligases, which tag proteins for degradation. The F-box motif is typically near the N-terminus and binds to a Skp1-like protein. The C-terminal portion contains protein binding motifs (Gagne et al. 2002). In SCF, Skp1 binds the F-box protein to a cullin protein. Other proteins, including RING (**R**eally **I**nteresting **N**ew **G**ene) domain proteins, also act as ubiquitin ligases to tag specific protein substrates (Willems et al. 2004). This led to the proposal of a model for SI based on specific protein degradation, which is discussed later.

4.4.3
Ubiquitylation and F-box Proteins Regulate Multiple Cellular Processes

A major function of polyubiquitylation is to target proteins for degradation by the 26S proteasome (Willems et al. 2004); it is also involved in degradation and disposal of misfolded and stress-injured proteins (Esser et al. 2004). F-box proteins also participate in other cellular processes (Kipreos and Pagano, 2000), utilizing non-SCF complexes. For example, the yeast F-box protein Rcy1p forms a complex with Skp1 and is involved in signalling and sorting between endosomes and the Golgi (Chen et al. 2005). Mono- or di-ubiquitylation is required for endocytosis and trafficking to the multivesicular body (MVB) and the vacuole (Hicke and Dunn 2003). It is also used to transport lysosomal enzyme precursors from the Golgi to the MVB pathway (Katzmann et al. 2004). Since changes in the pollen endomembrane system are associated with S-RNase based SI (Geitmann 1999), the role of F-box proteins in trafficking could be functionally important.

4.4.4
Ubiquitylation and SI: Models Proposing Proteolytic Inactivation of Non-self S-RNase

Several lines of evidence link ubiquitylation to S-RNase-based SI. The N-terminal and hypervariable regions of S-RNase interact with a putative pollen E3 ubiquitin ligase, SBP1 (Sims and Ordanic 2001). Key properties of SLF/SFB proteins are consistent with an ubiquitylation role. The SLF/SFB F-box is, as expected, very close to the N-terminus. The interaction of SLF

with SCF-like proteins in *Antirrhinum* also suggests that ubiquitylation plays a role in SI. Immunoprecipitation experiments indicate that it appears to interact with putative Skp1- and Cullin-like proteins (Qiao et al. 2004), but only when pollen and style extracts were mixed; complex assembly seems to require both pollen and pistil factors.

In the simple ubiquitylation model (Ushijima et al. 2004), SI results from degradation of non-self-S-RNase (Fig. 2). This model proposes that SLF actively inhibits non-self-S-RNase. S-RNases are non-specifically taken up into the pollen tube cytoplasm along with other ECM components. Once in the pollen tube, S-RNase interacts with a $SCF^{SLF/SFB}$ complex. In compatible pollinations, non-self-S-RNase is polyubiquitylated and degraded by the 26S proteasome complex. In incompatible pollinations, self-S-RNase is not polyubiquitylated and so persists, allowing for degradation of RNA by self-S-RNase and inhibition of pollen tube growth.

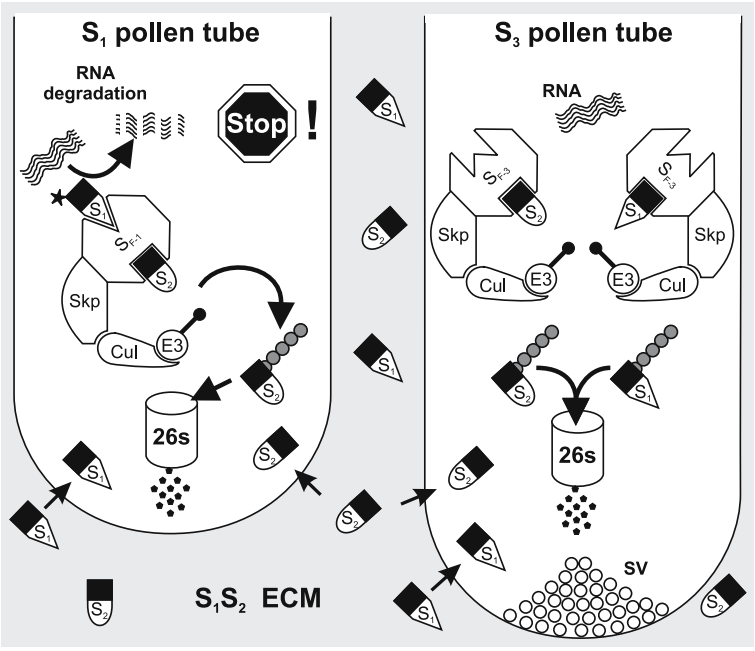

Fig. 2 Simple ubiquitylation model of S-RNase-based SI. Pollen tubes take up S-RNases non-specifically into the cytosol. SLF/SFB-S_1 interacts with both S-RNases present in the pistil. *Left*, (incompatible pollen) S_2-RNase is polyubiquitylated by $SCF^{SLF/SFB\text{-}S1}$ and targeted for degradation by the 26S proteasome. S_1-RNase binds *S*-specifically to $SCF^{SLF/SFB\text{-}S1}$ and is allowed to persist. S_1-RNase activity leads to pollen RNA degradation, and thus, tube growth inhibition. *Right*, (compatible pollen) both S-RNases are polyubiquitylated by $SCF^{SLF/SFB\text{-}S1}$ and targeted for degradation by the 26S proteasome. As no S-RNase activities persist, the pollen can grow. Key and symbols, as in Fig. 1

While there is some evidence for S-RNase ubiquitylation (Qiao et al. 2004a), this model may be an oversimplification. The model is not consistent with observations of large amounts of S-RNase in compatible pollen tubes (Luu et al. 2000) because it predicts degradation of nonself-S-RNase. The model also does not explain why non-S-RNase factors, known to be required for pollen rejection, are needed. To better elucidate the role of ubiquitylation in SI, two key components must be identified: a specific substrate for the $SCF^{SLF/SFB}$ complex and a specific E3 ligase-like protein that associates with the $SCF^{SLF/SFB}$ complex.

4.4.5 A Modified Inhibitor Model

An alternative model proposes that pollen recognition and inhibition reside on separate molecules (Fig. 3). While this "modified inhibitor model" is easier to reconcile with studies of the breakdown of SI in tetraploids and is supported by genetic studies in the Solanaceae (Kao and Tsukamoto 2004), there is no extensive biochemical support for it. In the version shown in Fig. 3, a general

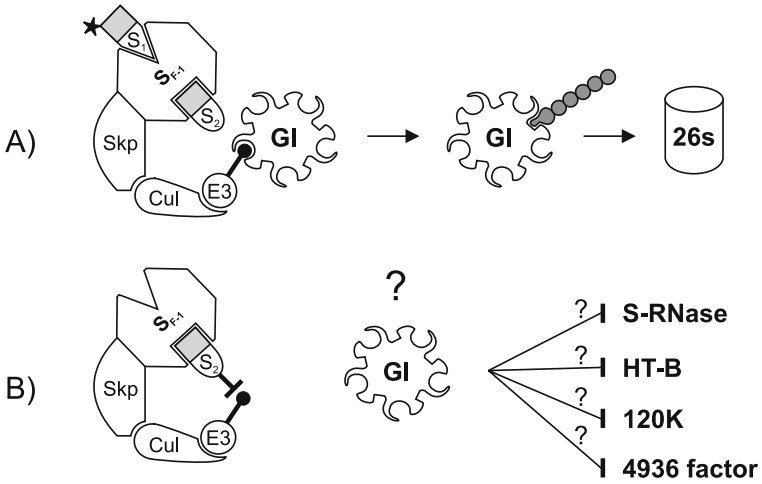

Fig. 3 Modified ubiquitylation model of S-RNase-based SI. **A** Incompatible pollination. $SCF^{SLF/SFB\text{-}S1}$ (Skp-Cul-F-box-E3 complex) interacts with both S_1- and S_2-RNase. The self interaction somehow inactivates the GI. One potential mechanism (shown here) for the inhibition is polyubiquitylation of GI by an $SCF^{SLF/SFB\text{-}S1(S1\text{-}RNase)}$ complex followed by GI's proteolytic inactivation in the 26S proteasome. When any required factor necessary for SI is inhibited, pollen can grow. **B** Compatible pollination. $SCF^{SLF/SFB\text{-}Sx}$ interacts with both non-self S-RNases. The non-self interaction inhibits the action of the $SCF^{SLF/SFB}$ E3 ubiquitin ligase complex, thereby allowing a hypothetical general inhibitor complex (GI) in pollen to persist. This GI can act on the S-RNase and other non-*S* factors, preventing their activity. Key and symbols, as in Fig. 1

inhibitor (GI) could act on any pistil factor required for pollen rejection. The $SCF^{SLF/SFB}$ complex inactivates the GI in an incompatible pollination, leaving both the S-RNase and non-*S* factors active. In a compatible pollination, the GI is active and inhibits the activity of S-RNase or non-*S* factors. In the modified inhibitor models proposed previously inhibition is explained as resulting from a non-*S*-specific GI directly binding S-RNase. However, the genetic data are consistent with any biochemical mechanism that prevents the cytotoxic action of S-RNase. Furthermore, this model allows for inhibition of targets other than S-RNase in compatible pollen tubes, such as HT-B or 120 K.

4.4.6 An Alternative Model

As mentioned earlier, the F-box protein Rcy1p has a different role, namely in endocytotic membrane trafficking. Such trafficking is vital to pollen tube growth, suggesting a possible alternative function for SLF/SFB. This opens up the possibility of an alternative model, which proposes that, during SI, vesicle trafficking in compatible pollen tubes is modulated by SLF/SFB so that S-RNase never reaches the cytoplasm in amounts sufficient to cause rejection. If S-RNase enters the pollen tube by endocytosis, then it may be selectively sorted to vacuolated regions of the pollen tube and remain isolated from the cytoplasm. The non-*S* factors could function to facilitate interaction between S-RNase and SLF. In SI, this interaction results in the release of S-RNase from a membrane-bound compartment. SLF, in turn, traffics non-self-S-RNase to metabolically inactive regions and self-S-RNase to the cytoplasm for its cytotoxic action. Support for this model, however, is based only on preliminary data showing that portions of S-RNase, HT-B, and 120 K in the style are associated with a membrane. Further testing is clearly needed.

There is still much that needs to be elucidated about the S-RNase based SI system. While the *S*-determinants are known, it is still not understood how they interact with each other to regulate pollen tube inhibition. The $SCF^{SLF/SFB}$ complex needs to be better characterized to determine its substrate(s). Only when a substrate is identified can the effects of the interaction between SLF and S-RNase be determined. Furthermore, the mechanisms for uptake, sorting, and trafficking need to be studied to determine how S-RNase gains access to the pollen tube cytoplasm, and how its activity is controlled. There are clear differences between the ubiquitylation mechanisms leading to 26S proteasome degradation and MVB trafficking (Hicke and Dunn 2003), so establishing the exact nature/role of ubiquitylation mechanisms in SI may clarify matters. Research has concentrated on investigating active mechanisms where pollen-*S* binds or degrades non-self-S-RNase. There has been little discussion of more passive routes to self-compatibility, such as sequestration of S-RNase away from its RNA substrate. A shift in emphasis will be helpful in evaluating potential mechanisms for controlling S-RNase cytotoxicity.

5
Cellular Mechanisms of GSI in Papaveraceae

Unlike S-RNases, the pistil-*S* gene products in *Papaver rhoeas* (named S-proteins) are not abundant proteins. They are specifically expressed in the stigma, which is the site of pollen inhibition. They are small ($\sim$ 15 kDa) secreted proteins without homology to any proteins with known function (Foote et al. 1994). Sequence comparisons between five stigma *S*-alleles revealed high polymorphism but no hypervariable "blocks" (just a single hypervariable amino acid in a predicted surface loop). Site-directed mutagenesis revealed several residues in this region that participate in pollen rejection (Kakeda et al. 1998). Because these proteins are small, secreted, and unique, it was proposed that they act as *S*-allele specific ligands that trigger a signalling cascade in incompatible pollen, and this was subsequently confirmed. The *Papaver* pollen-*S* receptors for the ligands have not yet been identified. Unlike S-RNase based GSI, the establishment of an in vitro SI system benefited analysis of *Papaver* SI at the cellular and biochemical level. Recombinant stigmatic S-proteins elicit an *S*-specific response in incompatible pollen that reproduces the in vivo response. Thus, additional non-*S*-linked pistil components are not required.

5.1
Calcium Acts as a Second Messenger in Papaver GSI

Cytosolic-free calcium ($[Ca^{2+}]_c$) is a key second messenger in intracellular signalling. To test whether the SI response in *Papaver* is mediated by $[Ca^{2+}]_c$ and S-proteins act as ligands interacting with a receptor on the pollen, calcium imaging was used. Almost instantaneous increases in $[Ca^{2+}]_c$ were detected after interaction between the S-proteins and incompatible pollen tubes, while none were detected during compatible interactions (Franklin-Tong et al. 1993). This established that Ca^{2+} signals SI in incompatible pollen. Unexpectedly, the $[Ca^{2+}]_c$ increases occurred in the shank of the pollen tube (Franklin-Tong et al. 1993, 1995). Ratio imaging of $[Ca^{2+}]_c$ confirmed these observations and established that the tip-focused apical $[Ca^{2+}]_c$ gradient dissipated within one minute, coinciding with cessation of pollen tube growth (Franklin-Tong et al. 1997). Further studies demonstrated that SI stimulates Ca^{2+} influx in the shank of incompatible pollen tubes (Franklin-Tong et al. 2002), establishing that extracellular Ca^{2+} contributes to the $[Ca^{2+}]_c$ increases.

5.2
SI in Papaver Stimulates Increased Phosphorylation of Pollen Proteins

Identification of SI-induced Ca^{2+} signalling led to investigation of downstream events, including phosphorylation of pollen proteins. Calmodulin and Ca^{2+}-dependent protein kinases frequently transduce Ca^{2+}-signals via

protein phosphorylation. Labelling pollen with ^{32}P ortho-phosphate enabled identification of several cytosolic pollen phosphoproteins exhibiting increases in phosphorylation specifically in incompatible pollen after SI (Rudd et al. 1996).

p26 was identified as a 26 kDa cytosolic pollen protein that exhibits SI-specific increases in phosphorylation within 90 seconds after interaction with incompatible S-proteins. Kinase inhibitor studies suggest that a CDPK (**c**alcium **d**ependent **p**rotein **k**inase) is involved (Rudd et al. 1996). p26 comprises two proteins, Pr-p26a and Pr-p26b, which are soluble inorganic pyrophosphatase (sPPase) homologues (de Graaf et al., personal communication). sPPases catalyze hydrolysis of pyrophosphate (PPi) to orthophosphate (2Pi). Their activity is essential for making biosynthetic processes thermodynamically favourable (Cooperman et al. 1992). Therefore, changes in sPPase activity and the PPi/Pi equilibrium can have a dramatic effect on metabolism. Because pollen tubes are fast growing cells, p26 sPPase may function in pollen tubes by affecting the rate of long chain polymer biosynthesis involved in tip growth. As increases in $[Ca^{2+}]_c$ reduce Pr-p26a/b sPPase activities, this suggests a possible functional involvement for sPPase in the inhibition of incompatible pollen tube growth, as sPPase activity is predicted to be essential for growth.

MAPKs (**m**itogen **a**ctivated **p**rotein **k**inases) are often associated with stress responses in plants (Innes 2001). As SI might be regarded as a stress response, the possible involvement of MAPKs in SI was investigated. A pollen phosphoprotein, p56-MAPK, which exhibited increased MAPK activity in incompatible, but not compatible, pollen tubes, peaking at 10 minutes after SI induction, was identified (Rudd et al. 2003). An intriguing feature of the p56-MAPK activation is its timing. Its activity peaks several minutes after the arrest of pollen tube growth, which means that p56-MAPK is unlikely to play a role in the initial rapid arrest of tube growth. However, because MAPKs can induce programmed cell death (PCD) in plants (Yang et al. 2001), p56 MAPK may be involved in a PCD signalling cascade in incompatible pollen (Sect. 5.4).

5.3 The Pollen Actin Cytoskeleton is a Target for SI in Papaver

As the actin cytoskeleton is highly dynamic and plays an essential role in pollen tube growth (Staiger 2000; Yokota and Shimmen, this volume), it was investigated whether actin was affected by SI. SI induces rapid and dramatic alterations in F-actin in incompatible pollen tubes within one minute of SI induction; compatible pollen tube F-actin was unaffected. By 5–10 minutes, longitudinal F-actin bundles largely disappeared, and the remaining F-actin had a fine, speckled appearance. Later, F-actin forms "punctate foci" which accumulate for several hours (Geitmann et al. 2000). Quantification of F-actin

content in pollen during SI demonstrated that SI induces very rapid, massive, and sustained F-actin depolymerization in incompatible pollen. Drugs that increase $[Ca^{2+}]_c$ in pollen tubes also triggered reorganization and quantitative reductions in F-actin (Snowman et al. 2002). Thus, the pollen F-actin cytoskeleton is a target for very early SI signals in incompatible *Papaver* pollen.

The dynamic nature of the actin cytoskeleton depends on localized activity of actin-binding proteins (ABPs), including several identified in pollen tubes (see Yokota and Shimmen, this volume). PrABP80, a Ca^{2+} regulated ABP, was identified as having a potential role in SI-mediated F-actin depolymerization. Mass spectrometry sequence analysis and in vitro kinetic actin polymerization assays identified PrABP80 as a gelsolin. Since PrABP80 exhibits potent Ca^{2+}-dependent severing activity, it may mediate the SI-induced F-actin depolymerization. Experimental data support synergistic action of PrABP80 and profilin on F-actin depolymerization (Huang et al. 2004). Thus, a potential mechanism linking SI-induced $[Ca^{2+}]_c$ increases to actin depolymerization has been identified. Such a mechanism would be predicted to rapidly inhibit incompatible tube growth, suggesting that actin depolymerization plays a key role in pollen tube inhibition in SI.

5.4 Programmed Cell Death (PCD) in Pollen is Triggered by SI

PCD is a highly conserved process used to remove unwanted cells in eukaryotes. Possible examples of PCD induced by external stimuli have been reported in plants (van Doorn and Woltering 2005). Several classic PCD markers have been identified in plant systems (Danon et al. 2000) and were used to investigate whether PCD is involved in the SI response in incompatible *Papaver* pollen tubes.

DNA fragmentation is a common marker for PCD and, in animal cells, involves activation of caspase-3. Caspases are proteases activated during apoptosis; they cleave many substrates including endogenous nuclease inhibitors, resulting in fragmentation of nuclear DNA (van Doorn and Woltering 2005). Investigations demonstrated that *S*-specific DNA fragmentation is triggered in incompatible, but not compatible, *Papaver* pollen tubes (Jordan et al. 2000). DNA fragmentation was first detected at 4 hours and continued for at least 16 hours after SI induction. Pre-treatment of pollen tubes with DEVD, a caspase-3 inhibitor peptide, significantly reduced DNA fragmentation observed in incompatible pollen tubes and enabled growth in the presence of incompatible S-proteins. The nuclear DNA repair protein, poly (ADP-ribose) polymerase (PARP), is a classic substrate for caspase-3 activity. Extracts from SI-induced pollen were tested for PARP-cleavage activity. Only extracts from incompatible pollen tubes generated the classic 24 kDa PARP cleavage product. These results provide evidence for a caspase-3-like activity being activated during the *Papaver* GSI response (Thomas and Franklin-Tong 2004).

Release of mitochondrial cytochrome c (cyt c) is another feature of PCD (Yao et al. 2004). The release of cyt c after mitochondrial membrane depolarization triggers caspase-activation. Unlike compatible pollen, incompatible *Papaver* pollen showed large increases in cytosolic cyt c within one hour of SI induction (Thomas and Franklin-Tong 2004). Because $[Ca^{2+}]_c$ signals to SI, investigations examined whether increases in $[Ca^{2+}]_c$ influence PCD in *Papaver* pollen. Stimulating increases of $[Ca^{2+}]_c$ in pollen (by mastoparan or A23187) triggered cyt c release into the cytosol and a high PARP-cleavage activity. Addition of a Ca^{2+} channel blocker, La^{3+}, resulted in a significant decrease in PARP cleavage. These data suggest that SI-induced increases in $[Ca^{2+}]_c$ trigger cyt c release and caspase-3-like activity resulting in PCD. PCD provides a mechanism that ensures that inhibited pollen does not resume growth. Ultrastructural EM studies revealed gross alterations to mitochondria, Golgi bodies, and ER in incompatible *Papaver* pollen within one hour of SI induction (Geitmann et al. 2004). This is much earlier than any changes observed in S-RNase based SI. Some of these changes have been observed in cells undergoing PCD, providing further support to GSI in *Papaver* being a PCD response.

Figure 4 summarizes the current knowledge about the signals and targets triggered by GSI in *Papaver* pollen. Induction of SI presumably involves an interaction of the stigma S-protein with its cognate pollen S-receptor. This interaction causes a rapid influx of extracellular Ca^{2+} and increases in $[Ca^{2+}]_c$ in the pollen tube. The tip-focused $[Ca^{2+}]_c$ gradient dissipates and tip growth is inhibited. $[Ca^{2+}]_c$ transduces the SI signal, triggering several downstream events, namely actin depolymerization. Actin depolymerization during SI is probably mediated through the cooperative action of several $[Ca^{2+}]_c$-regulated ABPs. Within 1 hour the SI-induced PCD cascade is initiated, resulting in incompatible pollen being committed to death. Activation of the p56-MAPK occurs after tip growth inhibition and is a candidate for signalling to PCD. Inhibition of Pr-p26a/b sPPase may act as a fail-safe mechanism to stop tip growth.

Some of the major components that mediate GSI in *Papaver* pollen have been identified and point to the involvement of others. For example, it would not be surprising if Rho-GTPases involved in the regulation of polar tube growth also were involved in *Papaver* GSI signalling networks. The challenges for the future thus involve identifying additional components, elucidating how they interact, and determining what cross-talk is used during SI. Another important question to answer is whether these interlocking signalling pathways are redundant. The interactions between $[Ca^{2+}]_c$, actin alterations and PCD signalling cascades are currently being investigated. The p56-MAPK activation may cross-talk to these elements; evidence to support this is currently being pursued because there is evidence for MAPK involvement in F-actin alterations and PCD in other systems. Another key question is whether Pr-p26a is involved in this cross-talk network, or acts independently as a fail-safe

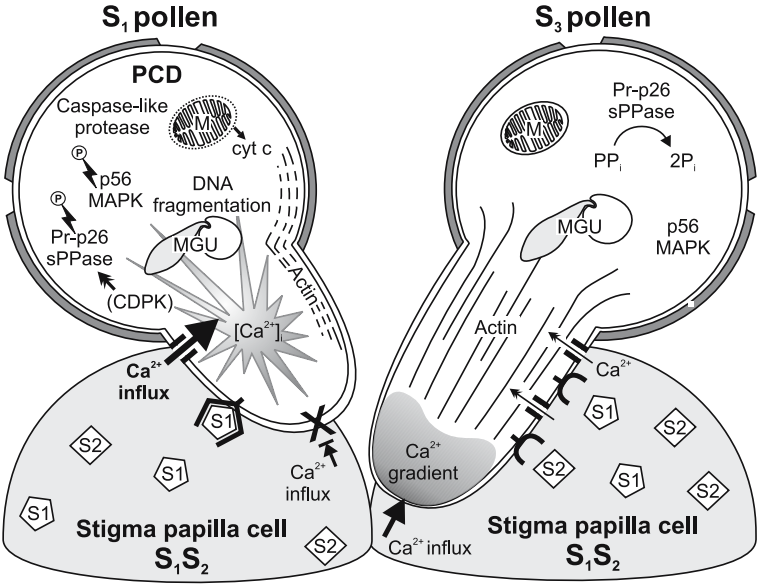

Fig. 4 Model of the cellular mechanisms involved in the poppy GSI system. *Right* (compatible pollen): high $[Ca^{2+}]_c$ at the tip, an intact actin cytoskeleton and active Pr-p26a sPPase. *Left* (incompatible pollen): an allele-specific interaction (binding of the S_1 protein to a S_1 pollen receptor) triggers an intracellular Ca^{2+} signalling cascade(s), involving large-scale Ca^{2+} influx and increases in $[Ca^{2+}]_c$ and loss of high apical $[Ca^{2+}]_c$. Two targets are rapidly modified: Pr-p26 shows an increase in phosphorylation and its sPPase activity is inhibited by $[Ca^{2+}]_c$, and F-actin is reorganized and depolymerized. Both cause rapid arrest of tip growth. p56-MAPK is activated and may signal to PCD. PCD is triggered, involving a caspase-like activity, cytochrome c leakage and DNA fragmentation. This ensures that incompatible pollen does not start to grow again

mechanism. There also remains the task of identifying the pollen receptor. This has been challenging; a gene closely linked to the stigma *S*-gene is currently being evaluated as a potential candidate. In the *Papaver* GSI system, pollen tube growth has a number of fundamental processes that can be inhibited by a simple signal from the stigma. *Papaver* seems to go to any lengths to prevent self-fertilization.

6 Perspectives

Despite marked differences between the two GSI systems, there are a number of common elements. They both must have the same molecular evolutionary pressure to locate both *S*-specificity genes at the same locus. Both have pistil signals that interact with pollen and since the pistil can exploit only

a limited number of "weak points" in interactions with the pollen, the underlying mechanisms involved in regulating pollen tube growth are likely to be similar. Therefore, any SI system has a limited number of variables that it can interfere with to inhibit tube growth. Although we say "limited", the regulation of pollen tube growth is complex and involves a large number of components. Thus, there may be a relatively large number of potential mechanisms that SI systems can employ. Here, we have identified multiple regulatory pathways ranging from ubiquitylation and processes dealing with the processing of cytotoxic molecules in S-RNase-based GSI, to phosphorylation, Ca^{2+} signalling, MAPK cascades, cytoskeletal rearrangement, complex signalling networks, and PCD in *Papaver* GSI. Both systems may have evolved from mechanisms targeting foreign invaders, but they have clearly used different strategies. S-RNase-based pollen rejection may be mechanistically related to cytotoxicity of binary toxins, such as ricin. *Papaver* appears to have derived its SI system from a pre-existing cell–cell communication system that leads to PCD.

A notable difference between the two GSI systems is the speed of the response. This may be related to differences in pistil morphology; *Papaver* has no style, so rapid inhibition is probably advantageous. Another major distinction between these two SI systems is that *Papaver* pollen contains an intact self-destruct mechanism just waiting for the right "trigger". The S-RNase based system, in contrast, uses pollen-*S* to overcome a cytotoxic barrier in order to allow successful fertilization, with self-S-RNase inhibiting the pollen's defences to the cytotoxic action.

One question worth asking, but beyond the scope of this discussion is: Why are there such completely different GSI systems? Presumably, SI is evolutionarily important as it has been selected several times independently. This suggests that any mechanism that interferes with pollen hydration, germination, or tube growth could potentially be selected as an SI mechanism. There is, therefore, a huge scope for different mechanisms and genes to be involved in SI. Which leads one to ask: Are there other SI mechanisms? The answer is clearly "yes". The multi-locus GSI systems (e.g., S- and Z-) in the grasses are likely to be different, as are the other single-locus SSI systems. Furthermore, although heteromorphic SI has not been examined at the molecular level, this probably uses biochemical mechanisms to back up the morphological barriers.

Looking ahead, future investigations are likely to involve a significant amount of cell biology. This should provide valuable insights into the physiology of SI mechanisms. A major challenge will be to perform experiments in vivo to establish whether the mechanisms proposed are correct.

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Extracellular Guidance Cues and Intracellular Signaling Pathways that Direct Pollen Tube Growth

Mark A. Johnson[1] (✉) · Elizabeth Lord[2]

[1]Dept. of Molecular Biology, Cell Biology, and Biochemistry, Brown University, 69 Brown St., Providence, RI 02912, USA
mark_johnson_1@brown.edu

[2]Department of Botany and Plant Sciences, University of California, Riverside, Berkeley, CA 92521-0124, USA

Abstract Fertilization in flowering plants requires that a pollen tube deliver two sperm to the female gametes, which develop in ovules buried deep within floral tissues. The tube germinates on a receptive stigma and enters the style where it grows rapidly in a nutrient-rich extracellular matrix secreted by cells of the transmitting tract (Lord 2003). Subsequently, it enters the ovary where it continues to grow on the surface of cells while targeting an individual ovule. Inside the ovule, the pollen tube immediately encounters the haploid synergid cells and continues to grow through the filiform apparatus, a specialized cell wall that forms at the basal junction of the two synergids. The journey ends when the tip enters one of the two synergids and bursts.

How does the pollen tube navigate these diverse environments within the pistil to reach a precise cellular target? Recently a great deal of progress has been made toward defining the sources of signals that direct specific stages of the pollen tube journey and toward identifying molecules that direct tube growth. However, our understanding of how the tube changes direction of growth in response to signals presented by floral cells along its path is still limited. For example, no pollen tube receptors have been identified for any of the extracellular guidance cues identified thus far and consequently, it has not been possible to assign specific signal transduction pathways linking the floral environment to changes within the pollen tube that cause reorientation of the tip. Here we review the recent progress toward identification of extracellular guidance cues and highlight efforts to understand how the tube perceives and transduces these signals into changes in the direction of its growth.

1 Introduction

The male gametophyte, or pollen grain, develops in the anther from a microspore and consists of a vegetative cell that contains two sperm cells (Twell et al., this volume). The nucleus of the vegetative cell (or tube cell) and the two sperm, which together make up the male germ unit, migrate near the tip of the pollen tube as it extends toward an ovule (Twell et al., this volume). The

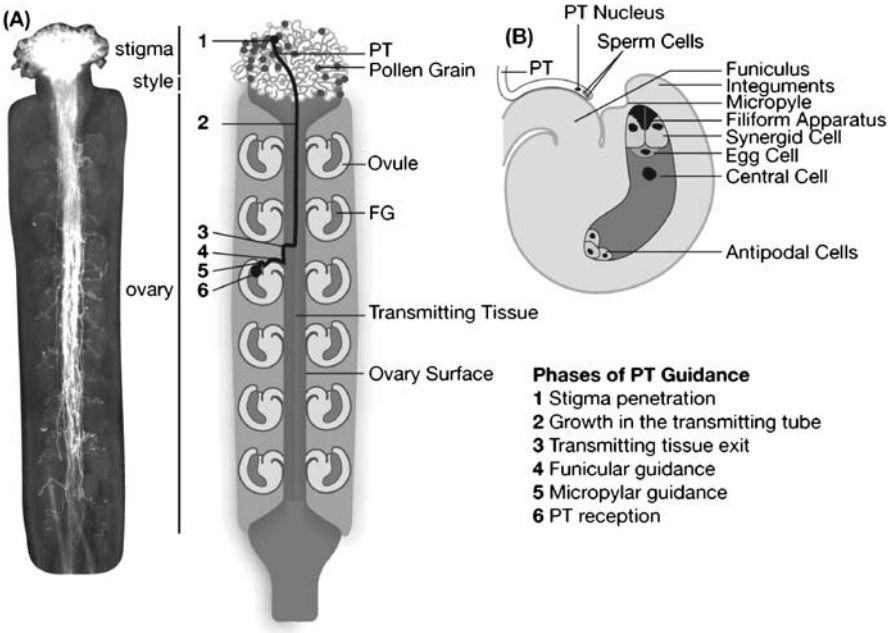

Fig. 1 Phases of pollen tube guidance. **A** The tube path is shown in an aniline blue stained Arabidopsis pistil (*left*) and in a schematic (*right*). **B** Critical cells and structures of the male and female gametophytes

female gametophyte, or embryo sac, develops within an ovule from a megaspore and most commonly consists of seven-cells (Yadegari and Drews 2004). The egg and the two synergids develop at the micropylar pole, three antipodal cells are located at the chalazal pole, the central cell is the largest cell and lies between the other two groups of cells. With the exception of the central cell, which is produced by fusion of a cell from each pole, each cell is haploid (Yadegari and Drews 2004).

Pollen tubes only encounter sporophytic cells on their way to the ovule and do not interact with gametophytic cells until they stop growing and burst within one of the synergids. However, there is growing evidence that pollen tube guidance is regulated by collaboration between sporophytic and gametophytic cells of the female tissue.

2 Major Models for Pollen Tube Guidance: Floral Architecture and Chemotropism

Two major hypotheses have been proposed to explain the precise growth of pollen tubes to ovules (Heslop-Harrison 1986, 1987). One holds that pistil

architecture dictates the path and that the pollen tube simply follows a mechanical trail of least resistance to the micropyle. Support for this model comes from anatomical studies of many species showing that the transmitting tissue of the style provides a defined environment for pollen tube growth that leads directly to the ovary. In the ovary, epidermal cells are arranged in files that lead to ovules and pollen tubes have been observed to grow between these files of cells (Shimizu and Okada 2000). In this hypothesis, the ECM of cells along the path would play a major role in guidance providing adhesive molecules that would keep the pollen tube on the prescribed path (Lord 2003), or in some cases may drive tube migration by pulling it along its path through a matrix-driven adhesion mechanism (Sanders and Lord 1989).

The chemotropic hypothesis posits that pollen tubes are directed to their target or along a series of intermediate targets by molecular guidance cues that change the direction of tube elongation. In this model, a target cell or cells produce molecules that either attract or repel pollen tubes. These molecules could be present in a gradient that continuously focuses the direction of the tube tip toward the highest concentration at its source. Such long-standing gradients were discussed to be difficult, if not impossible to attain (Lush 1999). Alternatively guidance cues may be expressed at a location that simply re-directs the tube tip, thereby changing the path of growth; in this case a continuous gradient may not be required and a point-source of the guidance cue may be sufficient (Mascarenhas 1975).

There has been a long history of debate over which of these two models best explain pollen tube guidance and at times the chemotropic model has been vigorously challenged (Heslop-Harrison 1986; Lush 1999). It is now clear from genetic analysis of tube guidance in *Arabidopsis* and in vitro guidance experiments in *Torenia fournieri*, that chemotropism plays a critical role in the final stages of tube growth and it is very likely that flowering plants use a combination of architectural constraints, ECM-derived adhesion molecules, and highly regulated production of chemotropic guidance cues to direct the pollen tube to the ovule.

3
Pollen Tube Guidance Comprises Multiple Steps and Requires Multiple Signals

A series of genetic experiments in *Arabidopsis* showed that tube guidance is a multiphase process in which early stages are controlled by sporophytic cells of the stigma, style, and transmitting tissue and the final stages are controlled by the female gametophyte. These experiments also provide critical support for a chemotropic component for tube guidance because some of the mutants analyzed do not disrupt floral architecture but disrupt tube guidance. Pollen

tube growth in *Arabidopsis* has been divided into the following phases (Huck et al. 2003; Johnson and Preuss 2002; Kandasamy et al. 1994; Rotman et al. 2003): (1) Stigma penetration, (2) Growth in the style and transmitting tissue, (3) Emergence from the transmitting tissue, (4) Funicular guidance, (5) Micropylar guidance and (6) Pollen reception.

Pollen tubes growing in pistils with homozygous mutations that disrupt ovule and FG development follow a chaotic path once they emerge from the transmitting tract and begin to grow on the ovary surface; tubes do not grow toward a funiculus, but grow on other surfaces including the ovary wall (Hülskamp et al. 1995). However, early stages of tube growth such as penetration of the stigma and rapid polar extension through the transmitting tissue of the style were not affected by these mutations. These experiments showed that tube guidance in the ovary is controlled by a mechanism distinct from that in the stigma and style and that the ovule and/or FG control pollen tube guidance in the ovary. Interestingly, the distribution of pollen tube exit points from the transmitting tract was also altered in these mutants, suggesting that ovule and/or the FG controls the position of tube exit from the transmitting tract (Hülskamp et al. 1995).

To determine whether sporophytic cells of the ovule or haploid FG cells direct pollen tube guidance following emergence from the transmitting tract, mutants were examined that block inception of FG development without affecting ovule development (Ray et al. 1997). In these mutants, all sporophytic diploid cells were normal, but half of ovules contained no FG. The path of wild-type pollen tubes growing in these mutant pistils was normal except they did not grow toward the funiculi of ovules that contained an aborted FG. This experiment showed that a functional FG was required for pollen tubes to target ovules.

Analysis of the *maa* mutants, which initiate but do not complete FG development, showed that growth of the pollen tube from the surface of the ovary to the micropyle can be divided into two phases (4 and 5) controlled by distinct mechanisms (Shimizu and Okada 2000). Unlike mutants that block initiation of FG development, *maa* mutants are able to attract pollen tubes to the distal regions of the funiculus, but these tubes fail to make the final abrupt turn required to enter the micropyle (Shimizu and Okada 2000). Therefore, micropylar guidance is controlled by a factor produced by FGs that are mature or near mature, while funicular guidance is controlled by a factor produced earlier in FG development.

In addition, these experiments showed that the FG could control repulsion of supernumerary pollen tubes. Ovules containing *maa* FGs attract multiple pollen tubes that remain stuck on the funiculus, whereas wild-type ovules attract a single pollen tube that grows up the funiculus and enters the micropyle. These results suggest either that the attractive signal is immediately quenched following tube entry into the micropyle, or that a second, repulsive signal is produced immediately following entrance (Shimizu and Okada 2000).

The final phase of guidance occurs when the tube stops growing and bursts. This stage is called pollen tube perception and was defined by analysis of FG-specific mutations called *sirene* and *feronia* (Huck et al. 2003; Rotman et al. 2003). These mutants attract pollen tubes into the micropyle, but these tubes do not burst, instead they continue to grow within the FG forming coils. Interestingly, *feronia* mutants attracted multiple tubes suggesting that entry of a tube into the micropyle is not sufficient to quench the attractive signal or initiate production of a repellant (Huck et al. 2003). These experiments clearly define a role for the FG in directing the final phases (4–6) of tube growth. Do the sporophytic cells of the ovule play any role in these phases? *ino* mutant ovules produce normal FGs, but fail to form the outer integument (Baker et al. 1997). The inner and outer integuments are layers of cells that encase the ovule; the micropyle is formed where these layers of cells converge. Interestingly, tube growth in pistils homozygous for *ino* is chaotic after emergence from the transmitting tract and is reminiscent of tube growth near ovules that completely lack an FG. This suggests that in addition to the FG, the outer integuments may be required for the funicular guidance step. It should be noted that the *ino* mutation also causes the micropyle to be oriented toward the base of the pistil rather than toward the stigma as in wild type. However, *ino* also causes the micropyle to be oriented toward the base of the pistil rather than toward the stigma as in wild type and this dramatic change in ovarian architecture could also account for the tube guidance defect observed in *ino*.

4
The Synergids are the Source of a Chemotropic Factor Directing Micropylar Guidance

The synergids appear to be specialized secretory cells (Higashiyama and Inatsugi, this volume). This feature led to the hypothesis that the synergids were a source for a chemotropic factor directing the pollen tube into the micropyle. This was directly tested using a semi-in vitro system developed for *Torenia fournieri* (Higashiyama et al. 1998; Higashiyama and Inatsugi, this volume) in which tubes grow through an excised style and out on to the surface of a culture medium before targeting the ovules placed on the surface of the medium. The FG of *Torenia fournieri* protrudes from the micropyle facilitating experimental manipulation and making it possible to analyze the role of the FG in tube guidance without intervening sporophytic cells. Laser ablation studies showed that at least one intact synergid was required for pollen tubes to target the FG (Higashiyama et al. 2001). The synergids were found to be the only FG cell type that is essential for attraction; ablation of the egg, central cell, or antipodals had no effect (Higashiyama et al. 2001).

Which phase of tube guidance as defined above for *Arabidopsis* is being analyzed in the *Torenia* system? In the *Torenia* in vitro system, tubes

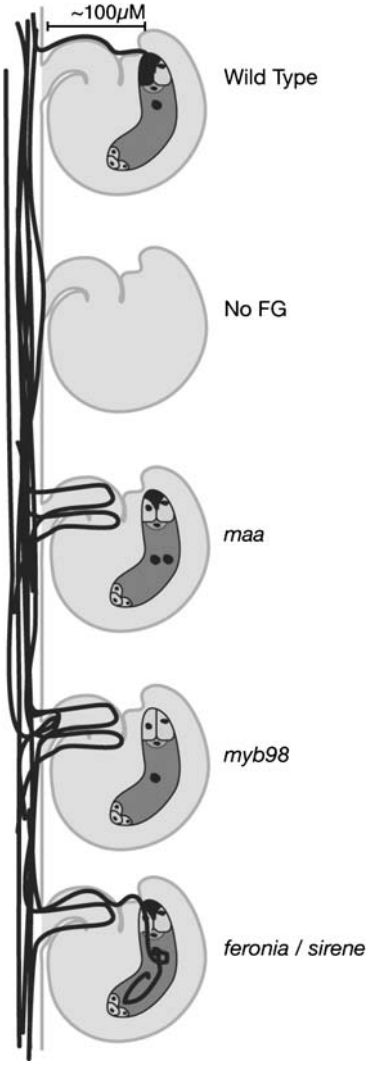

Fig. 2 Arabidopsis FG mutants define phases 3–6 of pollen tube guidance. Wild type ovules attract a single tube that enters the micropyle and bursts within one of the two synergids. Ovules that contain no FG do not attract pollen tubes to the micropyle and also show defects in the patterns of tube exit from the transmitting tissue. Maa mutants attract multiple tubes but these fail to enter the micropyle. Myb98 mutant attracts multiple tubes to the funiculus but fails to attract them to the micropyle. *Feronia* and *sirene* attract tubes that fail to burst within the synergids

grow directly to the filiform apparatus, a specialized area of cell wall at the basal junction of the two synergids; they do not contact sporophytic cells. In vivo, *Torenia* pollen tubes grow directly from the placental surface to

the filiform apparatus without contacting the funiculus (Higashiyama et al. 1998). Thus, *Torenia* appears not to have a phase analogous to funicular guidance in *Arabidopsis*. It is likely that the phase of guidance being interrogated in the *Torenia* system is analogous to the micropylar guidance phase in *Arabidopsis*.

Support for this idea comes from the recent analysis of the Arabidopsis FG mutant, *myb98* (Kasahara et al. 2005). All cells of *myb98* FGs develop normally except the synergids, which have a subtle and specific defect in the ultrastructure of the filiform apparatus. MYB98 is expressed specifically in the synergid cells and possibly functions as a transcription factor that regulates development of the filiform apparatus. *Myb98* FGs attract pollen tubes to the funiculus, but have defects in micropylar targeting. These data strongly suggest that the synergids are the source of the micropylar guidance cue in *Arabidopsis* and that the filiform apparatus is required for production and/or secretion of this chemotropic guidance cue (Kasahara et al. 2005). Biochemical purification of the active factor in *Torenia*, further analysis of *Arabidopsis* FG mutants, and analysis of genes that are specifically expressed in the synergids are promising approaches that could lead to identification of this molecule in the near future. Large-scale genetic analysis of the *Arabidopsis* FG has yielded a very interesting group of 18 *une* mutants that have apparently normal FG development but remain unfertilized (Pagnussat et al. 2005). Six *une* mutants were identified that failed to attract pollen tubes; further analysis of the genes disrupted in these mutants, which include genes of unknown function (UNE1, UNE4), a calcium binding protein (UNE14) and a small LEA protein (UNE15) will be an exciting area for future studies. Additionally, analysis of FG-specific gene expression has lead to the identification of a candidate micropylar guidance factor in maize, EA1 (Marton et al. 2005; Sect. 5.4).

5
Chemotropic Molecules Directing Tube Guidance

Chemotropic guidance molecules may be expected to meet the following basic set of experimental criteria: 1) the molecule should be present in a gradient with highest concentration at the target site 2) the molecule should have chemotropic activity (attractive or repellent) in an in vitro assay, 3) manipulation of the factor in vivo should have consequences for the direction of tube growth. Recently, a series of molecules have been postulated to function as chemotropic guidance molecules. Many of these have only been reported in the past few years and none of them yet fulfill all of these experimental criteria for a chemotropic guidance molecule. However, they all have features that are highly suggestive of chemotropic activity.

5.1 Chemocyanin

Lily pollen tubes gain access to the style through pores on the surface of the stigma and it was proposed that a chemotropic factor expressed on the stigma guides tubes that germinate on the surface of the stigma to these pores (Kim et al. 2004). A lily stigma protein preparation was shown to reorient tube growth in vitro. This preparation was further separated into multiple fractions by reverse-phase HPLC and only one of the fractions retained chemotropic activity. Analysis of this fraction showed that it contained one prominent protein with homology to a family of small basic proteins called plantacyanins and was called chemocyanin. Whether chemocyanin expression is focused around pores that lead to the hollow stylar canal has not been addressed but in vitro experiments suggest that chemocyanin functions by attracting tubes up a concentration gradient. Interestingly, chemocyanin activity in vitro was enhanced by inclusion of SCA (Kim et al. 2003), the small cysteine-rich adhesin that had already been shown to function in a complex with pectin to mediate adhesion of pollen tubes to a growth substrate in vitro (Mollet et al. 2000).

Plantacyanins have been found in many plants including *Arabidopsis* where the homolog is 52% identical to lily chemocyanin. Remarkably, recombinant *Arabidopsis* plantacyanin can reorient lily pollen tubes in vitro (Dong et al. 2005). A T-DNA insertion was identified in the *Arabidopsis* plantacyanin gene that reduced, but did not eliminate expression of plantacyanin. These mutants were fully fertile and any subtle defects in tube guidance were not reported. However, plantacyanin overexpression using the strong and ubiquitous cauliflower mosaic virus 35S promoter led to reduced fertility. When pistils overexpressing plantacyanin were pollinated with wild-type pollen, seed set was reduced by about 50% and some tubes were observed to coil around the stigma papillae and grow away from the style rather than toward it. However, many tubes were still able to penetrate the stigma, suggesting other defects within the ovary account for reduced seed set. In *Arabidopsis*, plantacyanin is prominently expressed along the path of the pollen tube and is particularly high in the transmitting tissue, suggesting it may function as a guidance molecule at a later stage of pollen tube growth. Plantacyanin is also expressed in the FG and it will be interesting to determine if plantacyanin plays a role at the terminal phases of tube growth as well.

Chemocyanin has chemotropic function in vitro and experiments in *Arabidopsis* show that manipulating plantacyanins in vivo can have consequences for tube guidance. Whether a gradient of chemocyanin forms near stigmatic pores in lily has not yet been tested and the biochemical function of plantacyanins in tube guidance remains to be elucidated. It will be interesting to determine whether the pollen tube expresses a receptor for plantacyanins.

5.2 GABA

The *pop2* mutant of *Arabidopsis* specifically disrupts pollen tube guidance. However, unlike the *Arabidopsis* mutants described above that define the phases of tube growth, it does not affect any female reproductive structures (Wilhelmi and Preuss 1996). Therefore, this mutation might directly affect the tube guidance system. When *pop2* pollen tubes grow in a *pop2-/-* pistil many of the tubes remain within the transmitting tract and those that exit the transmitting tract generally fail to enter the micropyle (Palanivelu et al. 2003; Wilhelmi and Preuss 1996). Therefore, this mutation disrupts phases 2–5 of tube guidance.

Pop2 mutant pistils accumulate ~ 100 fold more γ-amino butyric acid (GABA) than wild type because of a mutation in a transaminase that normally metabolizes GABA (Palanivelu et al. 2003). GABA is a four carbon ω-amino acid that has been shown to function in cell-cell signaling in many organisms and is best known as an inhibitory neurotransmitter in the human nervous system (Pinal and Tobin 1998). Immunofluorescence studies show that GABA is present in the *Arabidopsis* pistil along the tube growth path with peak concentration in the outer integument cells that surround the micropyle. This led to the proposal that GABA forms a chemotropic gradient that directs the tube to the micropyle. In *pop2* mutants, the abundance of GABA was higher throughout the pistil, and it was suggested that the GABA gradient directing tube growth to the micropyle is disturbed. *Pop2* is unique among guidance mutants described thus far because the mutant phenotype is only observed when the plant is self-fertilized. Wild-type pollen tubes navigate the *pop2* pistil without difficulty; perhaps this is because POP2 tubes retain GABA metabolic activity. Manipulation of GABA levels in vivo has dramatic consequences for tube guidance but chemotropic activity in vitro has not been demonstrated (Palanivelu et al. 2003). There are no obvious homologs of animal GABA receptors in the *Arabidopsis* genome (Palanivelu et al. 2003), so the molecular processes that mediate GABA signaling during pollen tube guidance remain unclear and novel approaches will need to be taken to identify and characterize GABA signaling components critical for pollen tube guidance in *Arabidopsis*.

5.3 TTS Proteins

Arabinogalactan proteins (AGPs) are encoded by a large gene family and display a diverse array of glycosylation patterns; importantly, they are among the most abundant proteins in the ECM of the transmitting tissue (Cheung et al. 1995; Wu et al. 1995). Pollen tubes incorporate AGPs into their cell walls as they extend and a synthetic molecule that binds AGPs has been shown to

disrupt tube growth in vitro (Mollet et al. 2002; Wu et al. 1995). These characteristics all suggest an important role for AGPs in regulating tube growth in the transmitting tissue. Transmitting tissue specific (TTS) AGPs from tobacco were shown to be more heavily glycosylated at the ovary end of the style and less so at the stigma end (Wu et al. 1995). When purified TTSs were placed in pollen tube growth medium they attracted tubes that had emerged from an excised tobacco style (Cheung et al. 1995). Attraction was mild and it is possible that tubes appear to grow toward TTS because they provide sugar and therefore stimulate growth. Transgenic tobacco plants expressing antisense versions of the TTS gene had reduced seed yield that resulted from reduced tube growth through the style; defects in the direction of tube growth were not observed. This suggests that TTS proteins are critical for tube growth in the style, but they may not be chemotropic guidance cues *per se*. TTSs may be required instead for rapid, highly polar growth in the transmitting tract that takes tubes to the ovary. It is thus possible that in the transmitting tissue tubes are not directed by chemotropic factors, but instead are just extending in a straight path that is defined by architectural features of the pistil and supported by nutritional factors such as TTS glycoproteins.

5.4 EA1

Maize *EA1* is expressed only by synergid cells and the egg and encodes a small protein of 94 amino acids with a predicted transmembrane domain (Marton et al. 2005). When EA1 was fused to GFP and expressed from its own promoter in transgenic maize plants, fluorescence was observed in the synergid cell walls of immature FGs. As ovules matured, fluorescence became more intense and spread into the six layers of nucellar cells that form the micropyle in the maize ovule. The *EA1* promoter alone drove reporter gene expression only in the egg and synergids,; thus, accumulation of EA1:GFP in the micropyle must be the result of secretion. Interestingly, EA1:GFP fluorescence did not appear to accumulate evenly in the cells that surround the egg and synergid. Instead EA1:GFP accumulated preferentially toward the micropyle leading to the suggestion that EA1 secretion and accumulation is tightly spatially regulated. This expression pattern is consistent with formation of an EA1 gradient around the micropyle.

Transgenic maize plants expressing either an *EA1* RNAi construct or an *EA1* antisense construct from the ubiquitin promoter had reduced seed set following self-pollination or when transgenic plants were used as the female in crosses with wild type (Marton et al. 2005). This effect was observed in a subset of transgenic plants; presumably those that disrupted EA1 expression, but this was not determined. Pollen tube guidance to *EA1* RNAi FGs was tested using in vitro pollination of ovules. In this assay, tubes entered the micropyle and burst within wild-type ovules 82% of the time. However,

this rate was reduced to 40–55% in ovules from two independent *EA1* RNAi lines. Pollen tubes were observed that grew past the micropyle in cases where ovule entry was unsuccessful. The EA1 expression pattern and the mild tube guidance phenotype in *EA1* RNAi plants are consistent with a function in the micropylar guidance phase. Therefore, EA1 meets two of the three criteria described above for a chemotropic guidance factor. Future studies will be aimed at determining whether EA1 functions directly as a chemotropic factor in vitro (Marton et al. 2005). It will also be interesting to clarify whether the mild impact on tube guidance is because EA1 expression was not completely eliminated in *EA1* RNAi plants, or whether this incomplete disruption of tube guidance indicates that there are redundant signals that direct pollen tubes to the micropyle in maize.

After fertilization, *EA1* mRNA was detected in the zygote for at least 45 hours but was not detected in 12-day-old embryos and EA1:GFP was eliminated rapidly from fertilized ovules (Marton et al. 2005). An interesting question will be to determine whether elimination of EA1 protein from the micropyle following fertilization is sufficient to prevent subsequent tubes from targeting the maize ovule or if a second repulsive signal is required.

5.5 Other Signals

While growing in the pistil, pollen tubes are exposed to many extracellular stimuli that could function as guidance cues. Several signals/molecules, including nutrients, have been reported to be involved in tube guidance including lipids, Ca^{2+} ions and cyclic nucleotides (Hülskamp et al. 1995; Malhó, this volume; Žárský et al., this volume). However, it is not clear if these molecules act as chemotropic signals.

Nitric oxide (NO) was recently suggested to reorient lily pollen tubes in vitro (Prado et al. 2004). In contrast to Chemocyanin and TTS, which behave as positive chemotropic factors, NO acted as a negative chemotropic factor, causing tubes to turn away from a point source. A gradient of NO was established by pipetting the NO donor, s-nitrosoacetylpenicillamine (SNAP), on to the surface of pollen growth medium. As tubes grew into the gradient, their growth rate slowed or stopped before the tip turned an average of 98° away from the point source. Interestingly, a mutant of Arabidopsis with a defect in NO production has reduced fertility, but tube guidance in this mutant has not been characterized (Guo et al. 2003). Rapid production of NO following entry of a tube into the micropyle could redirect subsequent tubes away from an ovule that is already being fertilized. It will thus be interesting to determine the sites of NO production within the pistil and to determine the path of pollen tubes growing within this and other NO synthesis mutants.

NO is known to regulate the production of the critical secondary messenger cGMP in animals. Sildenafil citrate causes accumulation of cGMP in

animal cells because it inhibits cGMP-degrading phosphodiesterases. Treatment of lily pollen tubes with sildenafil citrate sensitized the response to NO, suggesting that NO may also regulate cGMP production in pollen tubes. Interestingly, another cyclic nucleotide, cAMP has also been implicated in re-orientation of tip growth (Moutinho et al. 2001). Cyclic nucleotide synthesis is just beginning to be understood in plants (Malhó, this volume) and understanding the regulation of cyclic nucleotide signaling in pollen tubes will be an exciting area of future research.

6 The Search for Pollen Tube Receptors

How do pollen tubes recognize chemotropic guidance cues and transduce them into intracellular responses that result in changes in the direction of the tip? One hypothesis is that tubes express transmembrane receptors that specifically interact with guidance cues like those described above. Interaction between receptor and ligand is expected to initiate a signal transduction cascade within the tube that results in tip re-orientation and guidance (Malhó, this volume). It has been shown that localised $[Ca^{2+}]_c$ changes in the pollen tube apex control tube directioning (Malhó and Trewavas 1996). These changes may arise through asymmetric activity of putative Ca^{2+}-channels (Malhó et al. 1995; Sze et al., this volume). Preliminary evidence suggested the existence of low-voltage stretch-activated channels (Geitmann and Cresti 1998; Malhó et al. 1995) that could play a key role in the perception of guidance cues because of the multiple signalling pathways to which they respond (Ding and Pickard 1993).

Tremendous progress has been made in identifying intracellular factors that are crucial for tip growth (Malhó, this volume). For example, ROP proteins seem to act as a molecular switch that determines the site of cellular extension (Gu et al. 2005; Hwang and Yang, this volume). ROP modulates F-actin assembly and interferes with the dynamics of the tip-focused Ca^{2+} gradient, both of which are essential for tip growth and are thought to direct the flow of vesicles that ultimately results in polar tip extension (Malhó, this volume; Hepler et al., this volume; Yokota and Shimmen, this volume). Thus, changing the direction of tube growth may simply be a matter of changing the position/activity of certain proteins on the pollen tube plasma membrane. This has been demonstrated for several components like protein kinases, calmodulin, phosphoinositides and ROPs (Malhó et al. 2000; Malhó, this volume; Hwang and Yang, this volume; Žárský et al., this volume). Further characterization of these receptors for guidance cues and their modes of signal transduction within the pollen tube represent a very exciting challenge.

Genetic analysis of pollen tube-expressed genes required for guidance is an attractive experimental approach because it is not biased toward a pre-

conceived notion about how the tube perceives guidance cues and a thorough genetic analysis has the potential to identify genes that mediate multiple steps in signal transduction pathways. A loss of function mutation in a pollen tube-expressed guidance cue receptor putatively results in defective tube guidance that phenocopies loss of guidance cue production. For example, a tube that lacks the receptor for a micropylar guidance cue (perhaps EA1) would be expected to fail to enter the micropyle when grown in a wild type pistil the way wild type pollen tubes do when they grow toward an *ea1*- FG. Ideally, multiple mutants would be collected with defects in each of the specific phases of tube guidance described above and by identifying the disrupted genes in each group of mutants, hypotheses could be made about how the tube perceives cues that direct each phase of guidance. However, it is conceivable that such receptors act simultaneously as structural components of the tip growth machinery, and thus a mutation in their genes could result in the failure for pollen tubes to develop.

A large number of pollen tube mutants have now been characterized through a combination of reverse and forward genetic approaches (Twell et al., this volume; Guermonprez et al., this volume). Many of these affect tube germination and early stages of growth resulting in short tubes that fail to enter the ovary (Johnson et al. 2004; Lalanne et al. 2004; Guermonprez et al., this volume). These mutants have been useful for defining new mechanisms responsible for tip growth revealing a diverse set of biochemical processes that underlie tube extension. Several pollen mutants have also been characterized that do not affect germination, but disrupt tube growth; often these mutants have reduced growth rates that result in an inability to compete with wild-type tubes for access to ovules (Goubet et al. 2003; Johnson et al. 2004; Lalanne et al. 2004; Mouline et al. 2002; Schiott et al. 2004). Again, this group of mutants identified a diverse set of genes required for tube growth that include ion channels (Mouline et al. 2002; Schiott et al. 2004) and cell wall biosynthetic enzymes (Goubet et al. 2003). Despite growth defects, these mutant pollen tubes can target the ovules and are therefore able to respond to guidance cues along their growth path; thereby genetically separating the growth and guidance processes.

Pollen tube guidance mutants that have wild-type germination and growth rates, yet fail to target ovules, are rare. The *hapless* (*hap*) mutants are tagged with a pollen-specific marker gene that facilitates analysis of mutant pollen tube growth phenotypes within the ovary (Johnson et al. 2004). Out of 30 pollen mutants that were analyzed, 10 appeared to extend tubes that could grow the length of the pistil, yet these mutants showed reduced ability to target ovules. *hap1*, *hap18*, and *hap22* tubes failed to exit the transmitting tract; *hap11*, *hap26*, and *hap30* had relatively normal tube growth paths but were less likely than wild-type to enter the micropyle; and *hap2*, *hap4*, *hap24*, and *hap27* showed a chaotic growth path in the ovary and were often found growing on ovule surfaces where wild-type tubes do not grow (Johnson et al.

2004). So far, only a few of the genes disrupted in *hap* mutants have been identified and none of them appear to be similar to known receptors. Future work will be aimed at identifying more *hap* genes so that gene functions can be associated with specific defects in individual guidance steps.

Interestingly, pollen tube guidance mutants have yet not been found that completely block the ability of tubes to target ovules. This may suggest that there are redundant signaling mechanisms responsible for each phase of tube guidance and that no single gene mutation will completely disrupt guidance. Support for this idea comes from analysis of *ea1* mutants of maize and *myb98* and *pop2* mutants of *Arabidopsis* (Kasahara et al. 2005; Marton et al. 2005; Palanivelu et al. 2003; Wilhelmi and Preuss 1996). These mutants are proposed to disrupt production of chemotropic guidance cues, and all of them disrupt guidance: however, none of them completely block tube guidance.

A directed approach to identifying receptors for guidance cues is to study genes expressed by the tube that are homologous to known receptors. Microarray analysis of the 612-member receptor-like kinase (RLK) gene family in *Arabidopsis* showed that 10% are pollen expressed and that $\sim$ 90% of these are pollen specific (Honys and Twell 2003). This represents just one of several known receptor types expressed by pollen and illustrates the staggering potential diversity of signal transduction mechanisms in this cell. Functional characterization of members of the RLK gene family in tomato is well underway. LePRK1 and LePRK2 are pollen-specific RLKs that are localized to the surface of growing tubes (Muschietti et al. 1998). They both encode active kinases (Muschietti et al. 1998) and interact with each other at the pollen tube membrane; interestingly, this interaction and the phosphorylation of LePRK2 can be disrupted by incubation with a style extract in vitro (Muschietti et al. 1998; Wengier et al. 2003). This suggests that LePRKs may be involved in perception of extracellular growth regulators expressed by cells along the tube growth path.

Proteins that interact with the extracellular domain of LePRKs and are therefore putative ligands have been identified by yeast two hybrid screening of pollen [LAT52 (Tang et al. 2002)] and stigma cDNA libraries [LeSTIG1 and LeSHY (Tang et al. 2004)]. Loss of LAT52 function leads to pollen tube growth defects and LeSTIG1 has been shown to stimulate tube growth in vitro, suggesting that these two proteins may regulate growth via interactions with LePRKs. One interesting hypothesis is that the LePRK1/2 dimer switches ligands from LAT52 to LeSTIG1 upon contact with the stigma (Tang et al. 2004) and that this switch is critical for initiation of tube growth. One possibility to be tested is that LeSTIG1 is the active component of the stigma extract that causes LePRK1 and LePRK2 to dissociate and LePRK2 to become dephosphorylated.

To determine how signaling events at the pollen tube surface mediated by LePRKs are transduced into changes in tube growth, proteins that interact with intracellular domains of LePRKs were identified (Kaothien et al. 2005).

KPP is pollen-specific, phosphorylated in pollen, associated with pollen tube membranes, and its overexpression leads to defects in tip-localized actin dynamics that result in loss of apical polarity (Kaothien et al. 2005). All of these features implicate KPP in signal transduction events that regulate tube growth. Interestingly, KPP interacts with the cytoplasmic domains for LePRK1 and LePRK2 in vitro and this interaction does not require the kinase domains of either LePRK1or LePRK2. It is not yet known whether LePRK1 and/or LePRK2 mediate phosphorylation of KPP in pollen. In addition, it will be interesting to determine whether incubation with stigma extracts or LeSTIG1 alters the LePRK:KPP interaction or the phosphorylation status of KPP.

Analysis of LePRKs shows that taking a candidate-gene approach to the identification of receptors that sit atop pollen signal transduction pathways can be very productive. LePRKs have candidate ligands expressed by the pollen and stigma suggesting an autocrine/paracrine system for regulation of tip growth. Given the large number of candidate receptor genes expressed by pollen, more directed approaches will have to be taken to identify the specific receptor molecules that perceive guidance cues.

7
Are Guidance Factors Universal or Species Specific?

Pollen tube guidance cues and signal transduction pathways are being discovered in experimental model systems, and it will be extremely interesting to determine whether these systems are universal and function in all angiosperms or whether they are species specific. This is an important evolutionary question because diversification of tube guidance signals could represent a potent prezygotic barrier to interspecific fertility and may therefore be a means to achieve reproductive isolation following speciation. It is also an interesting question from an agricultural point of view because plant breeders would like to be able to make wider crosses between plants from different species but are sometimes hampered by incongruities in guidance systems.

There is ample experimental evidence indicating that important components of tube guidance mechanisms are species specific (Swanson et al. 2004). For example, pollen tubes of fellow Brassica family members like *Brassica sp.* and *Orychophragmus violaceus* fail to target *Arabidopsis* ovules in interspecific crosses (Kandasamy et al. 1994; Shimizu and Okada 2000). Pollen tubes germinate on the stigma and grow down the transmitting tract, but the majority of tubes failed to exit the transmitting tissue and those that did grew in the ovary in a fashion similar to tubes growing toward ovules lacking an FG (Ray et al. 1997; Shimizu and Okada 2000). Therefore, both the transmitting tract exit point signal (phase 3) and later signals mediating funicular

(phase 4) and micropylar guidance (phase 5) are likely to be species specific within the Brassicacae.

The guidance cues produced by *Torenia* and closely related genera are species specific in the in vitro guidance assay providing further evidence that the micropylar guidance signal is species specific (Higashiyama et al. 2003). While the chemical nature of the *Torenia* micropylar guidance cue is not yet known, the proposal that this signal is a polypeptide would accommodate species specificity due to rapid evolution and would also explain its limited effective range in vitro (Higashiyama et al. 2001). Species specificity could be achieved for a protein signal if the gene encoding the signal was subjected to positive selection following speciation and the receptors expressed by the pollen tube coevolved to more efficiently respond to the changed molecule. Interestingly, homologs for the proposed micropylar guidance cue from maize, the EA1 protein, were found only in rice, a fellow monocot, and alignment of EA1 proteins from maize and rice showed many amino acid changes especially at the N-terminus (Marton et al. 2005). It is possible that a small protein like EA1 serves as a micropylar guidance signal in all flowering plants, but rapid evolution during speciation has made EA1 analogs unrecognizable outside of grasses. Obvious EA1 homologs were not identified in the dicotyledonous *Arabidopsis* genome (Marton et al. 2005), however, the Arabidopsis genome encodes many small proteins that could represent the functional analog of EA1. Some crosses between maize and its ancestor teosinte are interfertile suggesting that micropylar guidance signals have not diverged since maize was domesticated (Baltazar et al. 2005); sequencing EA1 from teosinte and proximal relatives that are not interfertile will provide a test for the hypothesis that EA1 is rapidly diverging. In addition, identification of the synergid-derived signal that directs tube guidance in the dicot *Torenia* will reveal whether this signal could be an ancestor of EA1.

In contrast, a homolog of chemocyanin, the stigma guidance factor identified in the monocot lily, was identified in the dicot *Arabidopsis* (Dong et al. 2005). Furthermore, it was reported that plantacyanin from *Arabidopsis* can reorient lily tubes growing in vitro (Dong et al. 2005), suggesting that plantacyanins might be universal guidance cues. Overexpression of plantacyanin in *Arabidopsis* caused pollen tubes to coil and grow away from the style on papillae (Dong et al. 2005). These observations suggest that plantacyanins have a conserved function in orienting tube growth immediately after germination on the stigma. This conservation is particularly intriguing given the dramatically different architectures and chemical compositions of lily and Arabidopsis stigmas: In lily, tubes enter the style through pores in an exudate-rich wet stigma (Lord 2003); in *Arabidopsis*, tubes penetrate a papillar cell wall on a dry stigma (Elleman et al. 1992; Kandasamy et al. 1994).

Are small, structurally simple molecules, like GABA and NO universal guidance factors? Both of these molecules are produced by many organisms and are likely present in pistils of many if not all flowering plants. Despite

their ubiquity, a species-specific response could be mediated by quantitative differences in the amount of signal presented or in the sensitivity of the tube perception and response mechanisms. For example, co-evolution of the pollen tube and its host pistil following speciation could result in fine-tuning of GABA gradients and response pathways that would result in a species-specific response. Interestingly, when GABA levels are altered in *pop2* mutants, the tube growth path is very similar to that of *Brassica napus* or *Orychophragmus violaceus* tubes growing in an *Arabidopsis* pistil: most tubes fail to exit the transmitting tissue and those that do have defective funicular or micropylar targeting (Kandasamy et al. 1994; Palanivelu et al. 2003; Shimizu and Okada 2000).

The final phase of pollen tube guidance is reception (Phase 6), when the tube stops growing and bursts. In closely related, sympatric species without pre-pollination blocks to interfertility, this may be the last line of defense against interspecific fertilization. Characterization of the *feronia* and *sirene* mutants of *Arabidopsis* indicates that the FG produces a signal that controls this final phase and suggests that evolution of this signal could prevent tubes from other species from successfully delivering male gametes to the FG. Indeed, interspecific crosses in the genus *Rhododendron* are blocked after tubes enter the FG of the other species, but fail to stop growing and burst (Williams et al. 1986), much the same way wild-type tubes fail in *sirene* or *feronia* FGs (Huck et al. 2003; Rotman et al. 2003).

8 Perspectives

It is now clear that pollen tubes respond to chemotropic factors responsible for guidance in the pistil. Several phases of guidance have been delineated and multiple signals that act over different spatial ranges guide the pollen tube to the ovule. The sources of some of these signals within the pistil have been defined and chemotropic factors have been identified in a variety of angiosperm species using a combination of genetic, biochemical, and molecular approaches. The discovery of these molecules and their characterization marks tremendous progress in our understanding of guidance signals but many questions remain. Few of these molecules have satisfied all of the experimental criteria for a chemotropic guidance factor and none of them have been associated with pollen tube receptors or with the well-described signaling pathways within the tube that regulate tip growth. In addition, there are likely many more signals to discover and it will be very interesting to determine whether the signals defined thus far function in all plant species.

The field is now poised to define signal transduction pathways that link these extracellular guidance cues to intracellular networks that define, extend, and reorient the pollen tube tip. As these mechanisms are established

it will be exciting to determine the extent to which they are conserved among different plant species.

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Screening and Analysis of Pollen Tube Mutations

Hélène Guermonprez · Fabien Nogué · Sandrine Bonhomme (✉)

INRA, IJPB, Station de Génétique et d'Amélioration des Plantes, Route de Saint-Cyr, 78 026 Versailles cedex, France
bonhomme@versailles.inra.fr

Abstract Although the cytology of the cellular aspects of male gametophytic development has been very well described for several species, molecular factors, i.e., genes, involved in the different pathways occurring in this complicated process remain to be clarified. Developmental steps of male gametophyte are pollination, pollen tube germination on stigma, pollen tube growth and elongation through the style, pollen tube guidance and pollen tube reception by the ovule (McCormick 2004). For each stage, numerous cell–cell interactions and cues (biochemical, architectural and electrical) occur between the sporophyte cells (pistil) and the male gametophyte (Cheung 1996; Johnson and Lord, this volume). The study of mutants is a classical and now widely used approach to identify these cues and, consequently, to achieve a better understanding of molecular and biochemical mechanisms of pollen tube development.

1 Introduction

Classical mutations involved in plant reproduction can theoretically be easily detected, because homozygous mutants obtained by self-fertilization show fertility abnormalities. Sterile mutants harbouring shorter siliques or a lower siliques number compared to wild-type are easy to screen. The study of some of these reproduction mutations has allowed important insights about pollen tube development. However, a more direct strategy to describe the gametophyte molecular machinery is to create and analyse true gametophytic mutants. Gametophytic mutants are affected in the developmental step of plant haploid structure, i.e., male or female gametophytes, and their screening implies specific strategies. When such mutation affects only one gametophyte, the other can ensure transmission to the progeny, but no individual homozygous for the mutation can be obtained. Only heterozygous mutants are observed, producing 50% of wild-type gametophytes. However, in the case of male gametophytic mutant, half of wild-type pollen is sufficient to fertilize all the ovules, and heterozygous mutants therefore do not display a sterility phenotype. Moreover, phenotype analysis of affected tissue is complicated by the difficulties encountered in isolating gametophytes, and often needs extensive cell biology and cytology experiments. In terms of expected phenotypes on pollen tube development, a male gametophyte mutation can prevent or al-

ter pollen tube germination, or/and the pollen tube tip growth, and/or pollen tube guidance through the pistil, and/or gametes discharge. Finally, as pollen tubes are haploïd cells, anomalies in their development might also correspond to mutations affecting genes that function is essential, but not specific to the progamic phase, and only the haploïd status of the pollen makes the mutation lethal. This latter type of mutations is at least as much interesting as the others, as pollen tube offers the possibility to study specific processes in one living extending cell.

The first published pollen tube growth "mutants" were described in maize (Sprague 1933). The *wx* allele affected the gametophyte competitivity (estimated by measuring pollen tube length), that Sprague related to differential "establishment" performance (i.e., germination/attachment on silk). Twenty years later, the maize gametophytic factors Ga, whose ga recessive alleles lead to slower pollen tube growth, were reported by Schwartz (1950) and Nelson (1952). The level of cross-sterility was depending on the genotypes of the style (no fertilization on Ga^S/Ga^S, less fertilization on Ga/Ga). In these works, differences in pollen tube performance were deduced from the observation of distorted segregation of easy observable grains characters (e.g., colour).

Other reports of pollen tube growth mutations came from the analysis of embryo-lethal mutants. In *Arabidopsis*, following Ethane Methyl Sulfonate (EMS)-seed mutagenesis, Meinke (1982) observed higher rate of aborted vs wild-type seeds in the top half of the silique from two heterozygous embryo-lethal mutants. Those results were interpreted as possible evidence for slightly reduced pollen tube growth rate. Moreover, this work confirmed the suspected overlap of the sporophytic and gametophytic expression (Ottaviano et al. 1982) that has been largely corroborated in the following two decades.

In order to decipher the genetic and molecular control of pollen tube growth, as for other plant developmental processes, *Arabidopsis* is an attractive model: its genome size is small, the complete genomic sequence is available and numerous insertion mutant collections are accessible, permitting straightforward genetics. Moreover, cell biology of its reproductive process is very similar to that of many other plants, including crops, and is now very well described.

In this chapter, we will present the different strategies that have been developed to isolate so-called progamic mutations, affecting the pollen tube development, from germination to fertilization. We will not extend our report to self-incompatibility (SI), although works on SI brought much information on tip-growth key-signaling events (Barend et al., this volume). Similarly, the methods and tools used to analyze gametophytic mutants were recently reviewed (Johnson-Brousseau and McCormick 2004) and are discussed in Twell et al., this volume. Here we will focus on the different screening strategies that have been developed and the diversity of interesting mutant phenotypes and cloned genes that came out from these screens. We will include works that al-

though not aimed at studying pollen tubes, contributed also to increase the number of pollen-tube mutants.

2
True Male Gametophytic Mutants

2.1
Direct Screens Based on Gametophytes Observation

Direct visual screens have been developed in *Arabidopsis* to identify mutations affecting the male gametophyte, among fast-neutron (Chen and McCormick 1996), or EMS (Twell and Howden 1998; Park et al. 1998) mutagenized populations. These screens were designed to score early mutants on DAPI-stained mature pollen, a robust and 100% efficient protocol that allowed one to observe pollen grains morphology in microtitre plates (Chapter II). The cloning of the targeted genes in these mutants should bring interesting information on the regulation of pollen tube initiation, along with the analysis of other genes important for pollen cell wall and early pollen/stigma recognition steps (see below, Sect. 3). However, a reliable screen does not yet exist to reasonably allow the isolation of progamic mutants; in vitro pollen germination and tube growth of *Arabidopsis* is not reproducible enough for this purpose (Johnson-Brousseau and McCormick 2004).

2.2
Transmission Defect of a Marker

As direct visual screens were proved not appropriate to score for pollen tube growth mutations, alternative strategies have been designed, relying on the fact that gametophytic mutations affect pollen and/or embryo sac viability and function, and are therefore not transmitted to the self progeny by the mutated gametophyte. This leads to a segregation distortion for any trait linked to the mutation. If the mutation is linked to a reporter gene (e.g., resistance gene) brought through transformation, the segregation ratio can easily be estimated and compared to "normal" mendelian inheritance. This is the base of a forward genetic screen for gametophytic mutations that has been made possible by the increasing number of T-DNA or transposons insertion mutant collections in *Arabidopsis* (Fig. 1, Chapter II).

Feldmann et al. (1997) were the first to publish the results of a screen for non-mendelian inheritance of the Kanamycin antibiotic resistance trait brought by the T-DNA, in *Arabidopsis* transformants self progeny: Seven gametophytic mutations came out from their screen, affecting the pollen and/or the embryo sac viability. The mutants were defective in early steps of gametogenesis, prior to pollen tube development (Feldmann et al. 1997).

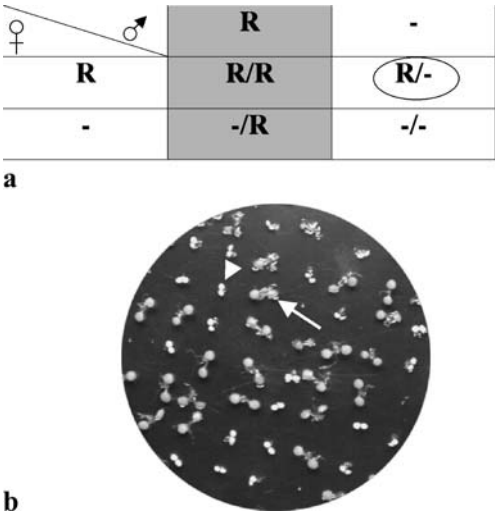

Fig. 1 Genetic screening of male gametophytic mutations. **a** transmission to the selfing progeny of a reporter gene (R) brought by T-DNA transformation. In this case, T-DNA insertion occurred at one locus, leading to male gametophyte lethality. T-DNA carrying gametes do not take part in fertilization (*shaded column*). Theoretically, a 1 : 1 segregation for the reporter gene is expected, instead of the mendelian 3 : 1 segregation, with only hemizygous progeny carrying the reporter (R/–, *circled*). Lethal female gametophytic mutation will lead to the same 1 : 1 ratio, with only –/R progeny carrying the reporter. Backcrosses on wild-type using the putative mutant as male or female will allow to estimate the transmission ratio through both gametophytes and thus to confirm the gametophytes lethality. **b** sowing on selective medium of the selfing progeny from an *Arabidopsis* male gametophytic mutant: half of the seedlings are resistant (R/–, *arrow*) while the other half are sensitive (*arrowhead*)

Scoring of other T-DNA transformant collections led to the cloning of two genes whose mutations result in abnormal pollen tube growth. (1) Three allelic mutants showing twisted and wider pollen tubes were affected in a single gene named *KINKY POLLEN* (*KIP*, Procissi et al. 2003). Homozygous progenies for the *kip* mutations were obtained, that showed shorter and wider root hairs. The *KIP* gene has putative homologs in most eukaryotes (Lobstein et al. 2003), and is similar to the *Arabidopsis SABRE* gene, whose mutation causes a dwarf phenotype (Aeshbacher et al. 1995). *Kip*/*sab* double mutant observation suggests related functions for both genes, with KINKY POLLEN protein having however a major role in tip growing cells, that remains to be identified. (2) The second gene that came out from the screen was named *POKY POLLEN TUBE* (*POK*, Lobstein et al. 2004) in relation to the very small (poky) size of mutant pollen tubes (Fig. 2). The encoded POK protein is 32% identical to the yeast Vps52p protein, involved in late Golgi retrograde vesicle transport (Conibear et al. 2003). Work is now in progress to establish its function in plant cells.

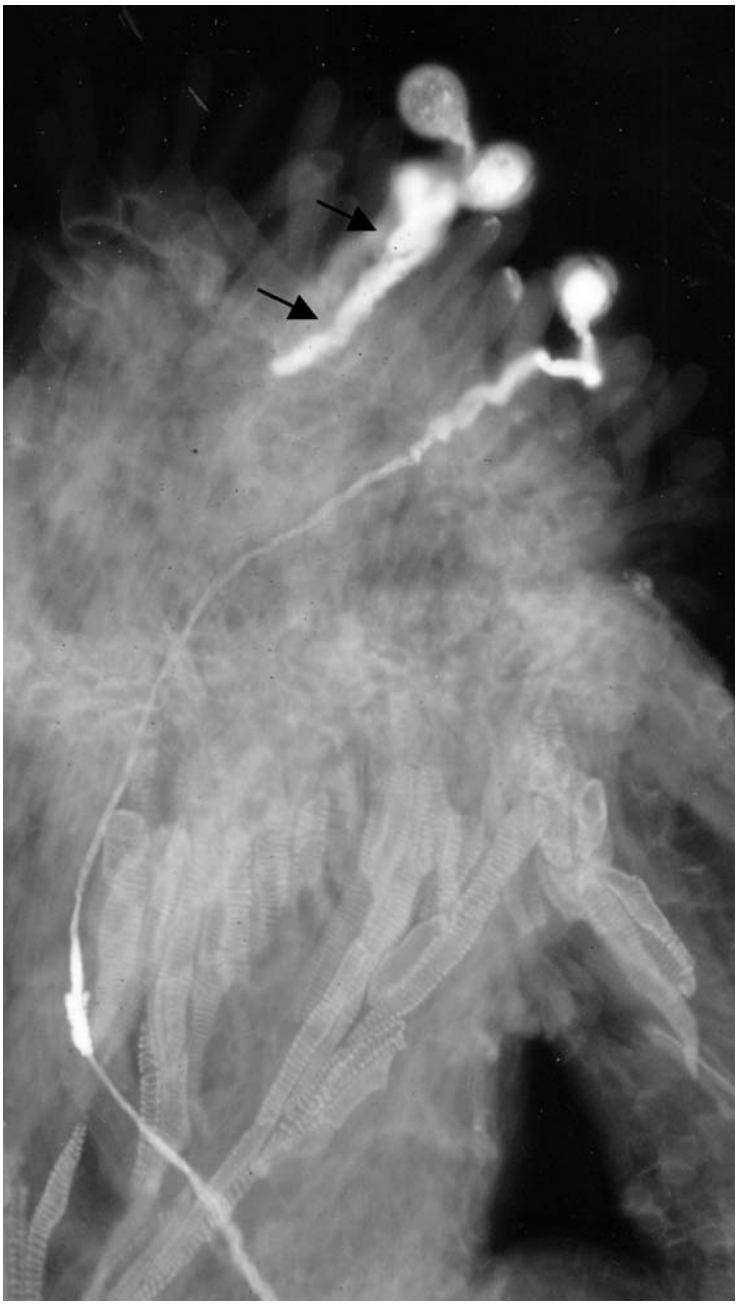

Fig. 2 In situ pollination assays, followed by aniline blue staining. Limited pollination (16H after pollination) using pollen grains from *pok* hemizygous mutant on wild-type pistil. Two pollen grains elongate very short pollen tubes (*arrows*), while a likely wild-type pollen tube has grown down into the ovary

In a slightly different approach, based on the use of the multiple marker chromosome *mm1*, Grini et al. (1999) reported the isolation of seven EMS mutagenized lines showing distorted segregation of markers nearby. One of those, called *mad4* for *male gametophytic defective*, showed visually normal gametophytes, but slower pollen tube growth in situ. The screening strategy in itself enabled the mapping of the mutation, but the gene corresponding to *mad4* has not been reported yet.

The number of mutations affecting pollen progamic development has significantly increased with the screen of *Ds* transposon insertion lines for distorted transmission ratios by Lalanne et al. (2004a). Twenty putative gametophytic mutants were selected from 3359 independent *Ds* insertion lines, among which 14 were defective in pollen tube germination and/or growth and/or guidance. Five *ungud* lines (from the name of a hermaphrodite Aboriginal snake deity) whose both female and male gametophytes were poorly transmitted, and nine *seth* lines (after the murderer of the Egyptian fertility god Osiris) strictly affected in the transmission *via* the pollen were described. Lalanne et al. introgressed the mutations into the *quartet1* background (Preuss et al. 1994). In the *qrt1* tetrad, callose is not degraded and microspores remain attached until mature stage, allowing to bring evidence for gametophytic effect through direct observation. Indeed, the tetrads from a gametophytic mutant should display two mutant pollen grains and two wild-type grains, and this can be monitored in vitro. While pollen germination is blocked in *seth6* and *seth7* mutants and reduced in *seth5*, pollen tube growth rate is slower in *seth8*, *seth9*, and *seth10*. Both steps are impaired in *seth1* and *seth2* mutants. The molecular cloning of Ds elements flanking regions allowed identification of interrupted genes as potentially key-elements in the progamic phase. Various functions such as cell wall biosynthesis, signaling, metabolism and protein anchoring have been highlighted (Lalanne et al. 2004a,b and Table 1).

Johnson et al. (2004) also scored for gametophytic mutations by looking for a resistance marker transmission defect, with the following features: (1) the screen was realised in the *qrt1* background and, (2) the transformation vector carrying the resistance marker included the LAT52:GUS sequence, monitoring the expression of the glucuronidase in pollen grains and pollen tubes (Twell et al. 1989). This provided an easy tool to distinguish, among selfing progenies showing a distorted ratio for the resistance marker, the hemizygous plants putatively carrying an insertion with an effect on the male gametophyte. Thanks to these markers, single insertion transformants were selected, and cases of large chromosomal changes (deletions or translocations) mimicking gametophytic mutations were discarded (Johnson et al. 2004). Out of 32 scored *hapless* mutations, 26 affected the progamic development: pollen tube growth was early arrested/slowered on stigma and/or style for 12 mutants, while growth or guidance was defective in the 14 remaining. The *hap1* mutant that has been functionally complemented is in-

Table 1 Cloned Arabidopsis male gametophytic mutations affecting progamic development:

Mutation	Affected step[a]	Encoded function[b]	Compl[c]	Reference
seth5	Pollen germination	Unknown transmembrane protein	yes	Lalanne et al. 2004a
seth6	Pollen germination	RPT2/NPH3-like protein	no	Lalanne et al. 2004a
seth7	Pollen germination	Ser/Thr protein kinase	no	Lalanne et al. 2004a
ungud6	PT development	Calcium-dependant Protein Kinase	no	Lalanne et al. 2004a
ungud9	PT development	Auxin induced protein (AIR9)	no	Lalanne et al. 2004a
ungud10	PT development	Fructokinase-like protein	no	Lalanne et al. 2004a
seth3	PT development	Arabinose-5-phosphate isomerase	yes	Lalanne et al. 2004a
seth1	Pollen germination, and tube growth	Phosphatidylinositol-glycan synthase C	yes	Lalanne et al. 2004b
seth2	Pollen germination, and tube growth	Phosphatidylinositol-glycan synthase A	yes	Lalanne et al. 2004b
pok	PT early growth	*vps52* (Vesicle trafficking)	yes	Lobstein et al. 2004
hap3	PT early growth	*SUC1* (Sucrose transporter)	no	Johnson et al. 2004
hap6	PT early growth	RibophorinII	no	Johnson et al. 2004
hap8	PT early growth	Unknown unique protein	no	Johnson et al. 2004
hap13	PT early growth	*MU1B* Clathrin adapter medium chain	no	Johnson et al. 2004
hap15	PT early growth	26S proteasome regulatory subunit S3	no	Johnson et al. 2004
kip	Tip-growth	Unknown protein	yes	Procissi et al. 2003
seth8	PT growth	GTP-binding typA-related protein	no	Lalanne et al. 2004a
seth9	PT growth	Alcohol dehydrogenase like protein	no	Lalanne et al. 2004a
seth10	PT growth	Unknown protein	no	Lalanne et al. 2004a

[a] PT: pollen tube; PT development: pollen germination, tube growth or guidance
[b] deduced from sequence homology of cloned gene
[c] "yes" indicates that genetic complementation experiments have been successfully done

Table 1 (continued)

Mutation	Affected step[a]	Encoded function[b]	Compl[c]	Reference
vgd1	PT growth	Pectin Methyl Esterase	yes	Jiang et al. 2005
hap1	PT growth and guidance	*Mago nashi* (cell polarity)	yes	Johnson et al. 2004
hap2	PT guidance	Unknown protein	no	Johnson et al. 2004
hap4	PT guidance	Ubiquitin extension protein1/ 60S ribosomal protein L40	no	Johnson et al. 2004
hap11	PT guidance	Mitochondrial ATPase δchain	no	Johnson et al. 2004

[a] PT: pollen tube; PT development: pollen germination, tube growth or guidance
[b] deduced from sequence homology of cloned gene
[c] "yes" indicates that genetic complementation experiments have been successfully done

terrupted in the promoter of a gene showing homology to the *Mago nashi* gene, that encodes a conserved protein associated with mRNA processing and translocation in animals. Insertion sites were identified for eight more *hapless* progamic mutants: gene expression, secretion, molecular transport and cellular energy production are functions potentially involved in pollen grain germination and tube growth (Johnson et al. 2004 and Table 1).

Although the genetic strategies permitted to identify several important genes, it did not bring the expected breakthroughs in the knowledge of gametophyte development genetic control. Other screens however gave access to genes essential for tip growth initiation and progress, including key-elements for latest steps prior fertilization.

3
Sporophytic and Female Gametophytic Mutations Leading to Altered Pollen Tube Germination, Growth or Guidance

While pollen tube growth mutants have so far never been directly monitored, visual screens for *Arabidopsis* mutants with lower seed set led to characterize mutations affecting pollen/stigma recognition phase and/or later steps of pollen tube's journey. Two types of mutations can be distinguished: (1) mutations affecting the diploid stage and leading to plant sterility (partial or full) when at the homozygous stage (hemizygous siliques are normal), and (2) female gametophytic mutations, affecting the haploid embryo sac.

3.1
Sporophytic Sterile Mutants

Chalcone synthase (CHS) deficient plants show a sterility that has been related to inhibited pollen tube germination and/or growth, both in maize *C2,Whp* double mutant (Coe et al. 1981), and in cossuppressed CHS transgenic petunia (Taylor and Jorgensen 1992). However, in *Arabidopsis* pollen function seems unaffected by flavonols deficiency (Ylstra et al. 1996).

Among *Arabidopsis* plants carrying sterility mutations (siliques of smaller size compared to wild-type) yet harbouring visually normal pollen, Preuss and co-workers isolated several mutants affected in pollen/pistil interaction (Wilhelmli and Preuss 1996; Chapter XI). These revealed the essential role of the protein and lipid-rich pollen coat for the very first steps of pollination later demonstrated by characterization of *Arabidopsis* mutants with altered exine and/or sporopollenin (Aarts et al. 1997; Ariizumi et al. 2003; Johnson and Lord, this volume).

The *vanguard1 (vgd1)* mutant was also scored because of a sterile (partial) phenotype among *Ds* insertion lines (Sundaresan et al. 1995). It is a male gametophytic mutant showing slower pollen tube growth when compared to

wild-type (Jiang et al. 2005). This phenotype explains the high frequency of sterile homozygous mutant progeny. The mutated gene *VGD1* encodes a protein homologous to pectin methyl esterase (PME), and estimations of its activity in pollen tubes indicated that it was lowered in the mutant.

In the double mutant *pop2pop3* first isolated by Wilhelmli and Preuss (1996), pollen tubes fail to adhere to the funiculus and thus to find the micropyle. The recent identification of the POP2 protein, a transaminase that degrades GABA (γ-amino butiric acid) suggests that this molecule plays an essential role in both pollen tube growth and guidance (Palanivelu et al. 2003). Moreover, in homozygous *pop2*/*pop2* later steps of guidance of the *pop2* pollen tubes are impaired possibly because of an inhibitory effect of elevated GABA concentration in the ovule surrounding tissue (Palanivelu et al. 2003).

3.2
Female Gametophytic Mutants Resulting in Disturbed Pollen Tube Development

In the *magatama* (*maa1* and 3) female gametophytic mutants, the embryo sac development is prematurely arrested (unfused polar nuclei), and if *maa* pollen tubes reach the ovule, they however fail to penetrate the micropyle (Shimiku and Okada 2000). The authors distinguished a micropyle guidance from a funiculus guidance, both controlled by putative diffusible female gametophytic signals. The targeted *MAA* genes remain to be identified. In contrast, in the *gfa2* female gametophytic mutant, where polar nuclei do not fuse and synergid cells do not undergo cell death, pollen tubes are normally guided until the micropyle. However, they do not discharge their content as no fertilization occurs (Christensen et al. 2002). The *GFA2* gene encodes a protein that is targeted to the mitochondria, shows homology to chaperone proteins, and was suggested to play a role in plant cell death.

Following γ-ray irradiation, other putative mutants affected in the pollen tube discharge and fertilization were scored among progenies that showed abnormal seed set, though normal pollen and embryo sac (Rotman et al. 2003). Live video-imaging of the *sirène (srn)* female gametophytic mutant showed that, unlike wild-type, *srn* pollen tubes do not stop growing inside the embryo sac and failed to deliver the male gametes. Concomitantly, the *feronia* female gametophytic mutant issued from a transposants collection was described as showing the same interesting phenotype (Huck et al. 2003). Unfortunately, the identity of mutated genes has not been revealed yet.

4
Reverse Genetics

Testing the potential involvement of plant homologues of known proteins in pollen tube growth has been another useful and successful strategy. To test

the function of these candidate genes implies the ability to create loss-of-function mutations. Methods have been developed for the identification of target-specific gene disruptions in large populations of insertion mutants and in the last few years, genome wide collections of sequence-indexed insertion mutants have been made available. This has turned the problem of obtaining a gene knockout into an *in silico* procedure for more than 70% of *Arabidopsis* genes (Alonso et al. 2003; Østergaard and Yanofsky 2004). An additional way to find a loss of function mutation in a candidate gene is the use of the TILLING technique (Targeting Induced Local Lesion in Genome). The TILLING is a general reverse-genetics tool, which combines random chemical mutagenesis with PCR-based screening to identify point mutations in a genomic region of interest (Henikoff et al. 2004).

4.1 Reverse Genetic Analysis Using Mutants

4.1.1 Functional Analyses of Candidate Genes

Calcium and calmodulin (CaM), an ubiquitous multifunctional calcium sensor in all eukaryotes, are known to play a crucial role in pollen tube development (Chapter I). Golovkin and Reddy (2003) isolated three closely related CaM-binding genes (*NPG1, NPGR1, and NPGR2*) from *Arabidopsis* and showed that only *NPG1* has a pollen specific expression. To analyze the function of NPG1, the authors isolated a mutant in which *NPG1* is disrupted by a T-DNA insertion. A distorted segregation ratio of the T-DNA insertion and the inability to produce homozygous plants, suggested that the mutated gene is not transmitted through the male gametophyte. Using the *quartet1* background the authors have shown that *npg1* pollen tube germination is impaired. Those results proved that NPG1 has an essential role in pollen germination.

Other key actors of calcium signalling are calcium pumps. Schiott and coworkers (2004) have described T-DNA insertion mutants in the *ACA9* gene, belonging to the autoinhibited Ca^{2+} ATPases (ACA) calcium pump that are predicted to be activated by Ca^{2+}/calmodulin. Disruption of the *ACA9* gene leads to non-mendelian segregation of homozygotes (Schiott et al. 2004) and an unequal distribution of seeds within the silique, with 96% of the seeds located in the upper half. Reciprocal crosses analysis between *aca9* and wild-type led to the conclusion that the *aca9* mutant is partially male sterile, and in vitro and in vivo studies showed that mutant pollen displayed a reduced tube growth potential and a high frequency of aborted fertilization (Schiott et al. 2004).

Dynamic membrane trafficking has been shown to be essential for proper tip growth in plants and is controlled by multiple proteins. Dynamin-related

GTPases regulate a wide variety of dynamic membrane processes in eukaryotes. Kang et al. (2003) have investigated the role of ADL1C in *Arabidopsis* and have shown that this protein is essential to the formation and maintenance of the pollen cell surface and thus influences pollen tube tip growth. Syntaxins are a large group of proteins found in all eukaryotes, also involved in the dynamic of membrane trafficking *via* their role in fusion of transport vesicles to target membranes. Sanderfoot and co-workers (2001) have described two families of syntaxin in *Arabidopsis* SYP2 and SYP4. Using T-DNA or transposon (Ds/Spm) insertion-mutagenized collections they have isolated and characterized gene disruptions in two genes from each family *SYP21* and *SYP42*. The authors have shown that disruption of individual syntaxins from these families is lethal in the male gametophyte of *Arabidopsis* due to default in pollen germination. The exocyst, a complex of eight proteins, contributes to the morphogenesis of polarized cells in a broad range of eukaryotes (Chapter VI). Orthologs of the eight genes (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) have been found in *Arabidopsis* and *AtSEC8* is expressed in both pollen and vegetative tissues (Cole et al. 2005). Genetic studies of *AtSEC8* T-DNA mutants showed that the mutant alleles showed a strong male-specific transmission defect (Cole et al. 2005).

The use of insertion mutant collections is not restricted to *Arabidopsis*, and recently Gass and co-workers (2005) have used a reverse genetic approach in Petunia to study how primary metabolism meets the demands of the rapid pollen tube growth. Using transposon tagging in *Petunia hybrida*, the authors have isolated a null mutant in the pollen-specific pyruvate decarboxylase Pdc2, a key enzyme of the ethanolic fermentation pathway. Growth of the mutant pollen tubes through the style is reduced, and the mutant allele shows reduced transmission through the male, when in competition with wild-type pollen, leading to the conclusion that this enzyme is essential for proper pollen tube growth in Petunia.

4.1.2 Functional Analyses of Gene Families

The *Arabidopsis* genome contains a great number of genes belonging to large families and in order to attribute a function to the different members of a same family of proteins, systematic analysis of insertion mutants in the corresponding genes have been initiated.

Initiation of transcription mediated by RNA polymerase II requires a number of transcription factors among which TFIID is the major core promoter recognition factor. TFIID is composed of highly conserved factors which include the TATA-binding protein (TBP) and around 14 TBP-associated factors (TAFs). Recently, the complete *Arabidopsis* TAF family has been identified. To obtain functional information about *Arabidopsis* TAFs, Lago and co-workers (2005) analyzed a T-DNA insertion mutant for *AtTAF6*. Detailed histologi-

cal and morphological analysis showed that the T-DNA insertion in *AtTAF6* specifically affects pollen tube growth. Exploiting also an *Arabidopsis* T-DNA insertion mutant library, Mouline and co-workers (2002) have shown the predominant role of a K^+ channel of the Shaker family (SPIK) in pollen tube development. Using a transposon (Ds/Spm) insertion-mutagenized collection, Goubet et al. (2003) have characterized an *Arabidopsis* mutant in the gene encoding AtCSLA7 of the cellulose synthase-like A subfamily. Analysis of the transmission efficiency of the insertion indicated that AtCSLA7 is important for pollen tube growth.

Apyrases hydrolyze nucleoside tri- and diphosphates are highly active and have been found in all pro- and eukaryotic organisms examined for their presence. In animals, these enzymes have been shown to play important regulatory roles in the mediation of signaling events. Two apyrases *AtAPY1* and *AtAPY2* have been characterized in *Arabidopsis* (Steinebrunner et al. 2000) and T-DNA knockout lines for each apyrase have been isolated (Steinebrunner et al. 2003). The single lines *apy1-1* and *apy2-1* did not display a phenotype under the conditions tested by the authors. However, the generation of the double knockouts *apy1-1/apy2-1* shows that pollen not expressing any apyrase is unable to germinate. This work shows the importance of gene redundancy in pollen development and emphasizes the limits of the use of insertion mutant analysis for reverse genetics.

4.2 Reverse Genetics Analysis Using Gene Down Regulation

Another alternative for the functional analysis of candidate genes is the ability to down-regulate the gene of interest through antisense or co-suppression (Brusslan et al. 1993), or for the more recent refinements of these methods, using RNAi-based technology. RNAi allows for targeted down regulation of genes, and vectors such as pHANNIBAL have been developed in which inverted repeats of a gene sequence can be inserted (Wesley et al. 2001). In some case these techniques can circumvent the problem of function redundancy and researcher can now use the strategy of RNAi to silence simultaneously different genes of the same family (Bensmihen et al. 2005). However, these methods have several drawbacks, including the lack of stable heritability of a phenotype and variable levels of residual gene activity.

Muschietti et al. (1994) were the first to design antisense experiments with the pollen-specific tomato gene LAT52. Plants with reduced amounts of LAT52 mRNAs and protein showed smaller pollen grains segregating 1 : 1. In vivo pollination experiments suggested pollen tube growth arrest in the style, for a proportion of the transformants pollen. In order to elucidate the function of another pollen-specific gene from tobacco, *NTP303* (Weterings et al. 1992), de Groot et al. (2004) have transformed tobacco plants with *NTP303* co-suppression and anti-sense gene constructs and shown that the

kanamycin resistance trait, which was linked to the *NTP303*-silencing gene, was not transmitted through the male gametophyte. This indicated that lowering the transcript level of NTP303 and its family members interferes with pollen function. In vivo studies demonstrated that NTP303 and its family members are essential for normal pollen tube growth.

Inositol polyphosphates are pivotal intracellular signaling molecules in eukaryotic cells (Zárský et al., this volume). In order to investigate the physiological function of the *Arabidopsis* inositol polyphosphate kinase AtIPK2alpha, Xu and co-workers (2005) have generated transgenic plants expressing the *AtIPK2alpha* antisense gene under the control of its own promoter. Analysis of several independent transformants exhibiting strong reduction in *AtIPK2alpha* transcript levels showed that both pollen germination and pollen tube growth were enhanced in the antisense lines compared to wild-type plants. In addition, seed germination and early seedling growth was stimulated in the antisense lines, suggesting a general role of AtIPK2alpha in the regulation of plant growth.

SHY, a petunia pollen-specific gene identified in a screen for genes upregulated at pollen germination, encodes a leucine-rich repeat (LRR) protein that is predicted to be secreted. Guyon et al. (2004) generated transgenic plants expressing an antisense copy of the *SHY* cDNA in pollen. Primary transformants exhibited poor seed set, but homozygous lines could be identified. In these lines, tube growth was arrested at the apex of the ovary and the pollen tubes exhibited abnormal callose deposits throughout the tube and in the tips. The authors suggested that SHY might function in a signal transduction pathway mediating pollen tube growth.

More recently, de Graaf and co-workers (2005) have shown that altering RAB11 activity in pollen by expressing either a constitutive active or a dominant negative variant of Rab11b, a *Nicotiana tabacum* pollen-expressed GTPase, resulted in reduced tube growth rate, meandering pollen tubes, and lower male fertility. Those results confirm the essential role of Rab-GTPase for tip-focused membrane trafficking and growth at the pollen tube apex (Hwang and Yang, this volume).

Using together T-DNA mutagenesis and RNAi, Gu et al. (2005) have demonstrated the crucial role in *Arabidopsis* of another family of GTPases. The authors have shown the involvement of the Rho family GTPase protein AtROP1 effectors proteins, RIC3 and RIC4 (ROP-interactive CRIB containing proteins), in the control of actin dynamics and tip growth (see detailed description in Hwang and Yang, this volume).

4.3 Alternative Techniques for Functional Analysis of Candidate Genes

Other strategies aiming at modifying the level of expression of a particular gene in the pollen tube have been used to study the involvement of these

candidate genes in tip growth. One of them is based on the possibility of transformation of isolated pollen tubes (e.g., *via* particle bombardment of pollen tubes). Using this technique, Yang's group clarified the role of At-ROP1 protein in *Arabidopsis* pollen tubes (Fu et al. 2001; Hwang and Yang, this volume). The authors found that transient overexpression of AtROP1 disrupts the dynamics of the tip-localized F-actin present in tobacco pollen tubes during polar growth. Another GTPase, RAB2, regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies. Cheung and co-workers (2002) have shown by studying the effect of the transient expression of a dominant negative version of the tobacco *NtRAB2* gene, that RAB2 is essential to optimally support the high secretory demands of growing pollen tubes.

Recently, *via* transient overexpression of the *AFH-1* gene in pollen tube, Cheung and Wu (2004) have shown the role of the *Arabidopsis* formin AFH-1 protein in actin cable formation from pollen tube cell membrane.

Transient pertubation of expression of particular genes can also be obtained using modified oligodeoxynucleotides (Lewis et al. 1996). Moutinho and co-workers (2001) delivered oligodeoxynucleotides having phosphorothioate modifications in the three bases adjacent to each terminus to *Agapanthus umbellatus* pollen growing in vitro. These oligodeoxynucleotide probes, directed against conserved motifs of adenylyl cyclase, perturbed tip growth guidance suggesting that modulation of cAMP concentration is vital for tip growth. Using the same strategy, Camacho and Malhó (2003) have been able to reduce the expression of the *AuROP* gene from *Agapanthus* by loading in vitro growing pollen with modified antisense oligonucleotides complementary to the *AuROP* cDNA. These experiments revealed that perturbation of pollen tube growth can be accomplished by reducing the level of ROP proteins, confirming the role of this family of proteins in polar growth.

5 Perspectives

In his review on pollen tube growth in 1993 (Plant Cell), Mascarenhas expressed his hopes in the exploitation of "the power of genetics and tagged mutants (...) in the study of the male gametophyte". Twelve years later, there is no doubt that, thanks to genetics and mutant analyses, a large amount of data has been accumulated that contributes to build a model for molecular control of pollen tube development. The number of cloned genes from gametophytic (and sporophytic) mutants studies has significantly increased in the past years, and many scored mutants remain to be analysed. Moreover, numerous and novel data from transcriptome studies now allow to expand a decade of experience in *Arabidopsis* gametophytes genetics (see Twell et al.,

this volume). Mutant collections, first screened through forward strategies are now being exploited *via* reverse genetics, to investigate the role of each single component of complex processes (such as cell wall biosynthesis, vesicle trafficking, endocytosis, GTPase signaling, etc.). This optimistic view, however, needs to be tempered by the fact that gene redundancy often hampers the characterization of specific mutants. This might explain for example that no *Arabidopsis* mutant for pollen cytoskeleton components has been characterized so far. In terms of signaling, small peptides, proteins and other molecules present in the female tissues (Johnson and Lord, this volume) are presumably determinant, but related mutants are rare.

Other genes involved in pollen tube growth might be identified through the study of other tip growing cells. Plant root hairs also show tip growth, but after a diffuse elongation of the trichoblast. This might partly explain that among the high number of *Arabidopsis* root hair mutations isolated so far (Carol and Dolan 2002), hardly none leads to a specific pollen tube phenotype. For a couple of genes, however, mutant phenotypes have been observed on both tip growing tissues: the *KIP* gene which role is unknown and the *TIP1* gene, for which the null mutation affects both diffuse and tip growth, but firstly led to a strong root hair phenotype (Ryan et al. 1998). The encoded TIP1 protein is an S-acyl transferase that has homologs in yeast and human. Relying on yeast mutant complementation with the *Arabidopsis* gene, the authors suggested that it might play a role in cell polarity through regulation of vesicle traffic (Hemsley et al. 2005).

Finally, other eucaryotic cells extend through tip growth process, such as filamentous fungi hyphae and moss protonema. Hyphal mutants for cytoskeleton proteins have been described (e.g., Virag and Griffiths 2003; Schuchardt et al. 2005) and their study might provide clues to decipher pollen tube cytoskeleton regulation. In the moss model *Physcomitrella patens,* mutants can be screened for protonema growth defect, and this has proved a successful strategy (e.g., Harries et al. 2005). Moreover, its full genomic sequence and the high rates of homologous recombination in *Physcomitrella* offer the possibility to create targeted mutations in this species (Schaefer and Zrÿd 1997) and thus analyse mutant phenotype of any gene of interest, provided that the homolog is present in the moss genome.

Pollen tube development is one of the plant biological processes where species-specific signals might be expected. Indeed, tricellular pollen as observed in *Arabidopsis* is not the most frequent feature for land plants, and generative cell division most frequently occurs after pollination. Furthermore, two types of style and transmitting tract tissues are present among plants, leading to presumably different signaling for pollen tube journey. Recent publication contradicts Marton et al. 2005. This diversity of processes emphasizes the necessity to work not exclusively with *Arabidopsis*.

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Comparative Analysis of Biological Models used in the Study of Pollen Tube Growth

Tetsuya Higashiyama (✉) · Rie Inatsugi

Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo, 113-0033 Tokyo; CREST, JST, Japan
higashi@biol.s.u-tokyo.ac.jp

Abstract The mechanisms of pollen tube growth have been studied in a wide variety of plant species. Since the 1990s, with the explosion of molecular genetic analyses in *Arabidopsis thaliana*, most studies started to focus on this model plant. However, because of their particular characteristics, plant species other than *Arabidopsis* are still used to reveal physiological mechanisms and identify novel molecules relating to pollen tube growth, including, for example, lily, tobacco, *Nicotiana alata*, tomato, rice, maize, *Brassica* spp., corn poppy and *Torenia* (Table 1). Here, we designate all of these relatively common experimental plants as "biological models" for the study of pollen tube growth. These models sometimes provide a good first step in the identification of novel physiological mechanisms and molecules. As genome sequencing technologies become more advanced, the difficulty of performing molecular analyses in these biological models will decrease. Thus, a better understanding of these biological models will allow researchers to perform unique studies of pollen tube growth. In this chapter, we compare the characteristics of biological models, focusing on in vitro systems, to facilitate the use of these biological models for in vitro analyses.

1 Comparison of Fundamental Characteristics of Biological Models

Table 2 shows the fundamental characteristics of biological models used in the study of pollen tube growth.

Arabidopsis thaliana is now the most commonly used model plant. This species has many characteristics that are useful in the study of reproductive processes. In addition to the availability of molecular genetic tools for *Arabidopsis*, its small, simple pistil is useful for the observation of entire path of growth of a pollen tube (Hülskamp et al. 1995). In vitro culture of the pollen tube is also possible (Sect. 2). Thus, the molecular mechanisms underlying gametophyte development, pollen–pistil interactions, and pollen tube growth have are also being studied in *Arabidopsis*. The pollen is tricellular (Fig. 1), i.e., it consists of one vegetative cell and two sperm cells, and shows no self-incompatibility. Recent population genomic analyses have shown that *Arabidopsis* became a self-compatible plant about 320 000 years ago because of the selective sweep of a pseudo allele of *SCR* gene, *ΨSCR1* (Shimizu et al.

Table 1 Biological models used in the study of pollen tube growth*

Brassicaceae	Solanaceae	Liliaceae	Poaceae	Papaveraceae	Scrophulariaceae
Arabidopsis thaliana (*Arabidopsis*)	*Nicotiana tabacum* (tobacco)	*Lilium longiflorum* (lily)	*Oryza sativa* (rice)	*Papaver rhoeas* (corn poppy)	*Torenia fournieri* (*Torenia*)
Brassica campestris —	*Nicotiana alata* —	*Agapanthus umbellatus* (*Agapanthus*)	*Zea mays* (maize)		*Antirrhinum majus* (snapdragon)
Brassica oleracea —	*Lycopersicon esculentum* (tomato)				
	Petunia hybrida (petunia)				

* In addition to these commonly used species, various other plants have been used in the study of pollen tube growth, including the following genera (see also Sect. 13.1, below): *Impatiens*, *Portulaca*, *Camellia*, *Tradescantia*, *Gibasis*, *Trifolium*, *Lathyrus*, *Melilotus*, *Pisum*, *Medicago*, *Lotus*, *Prunus*, *Pyrus*, *Ipomoea*, *Secale*, *Gasteria*, *Ornithogalum*, *Helianthus*, *Plumbago* and *Alnus*. Common names are indicated in parentheses below the scientific name; both are used in this chapter

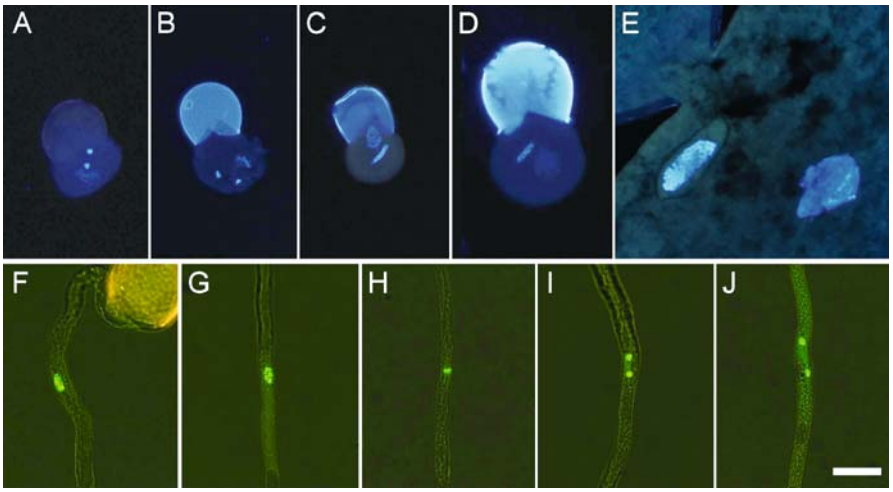

Fig. 1 Pollen grains stained with DAPI (**A** to **E**) and pollen tubes stained with SYTO11 (**F** to **J**). **A** to **E** Pollen grains of *Arabidopsis* (**A**), rice (**B**), tomato (**C**), *Torenia* (**D**) and lily (**E**) were observed after preparation by the squashing method of Sodmergen et al. (1992). Note that all panels are prepared in the same magnification of view. **F** to **J** Pollen tubes of *Torenia* were cultivated in a medium containing 300 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 100 mg/L H_3BO_3, 1% sucrose and 13% PEG 4000 at 30 °C for 4 h, and then they were stained by adding 1000-fold diluted (final) SYTO11 nucleic acid stain (Molecular Probes, Eugene, Oregon, USA). Nuclei of generative and sperm cells at various stages are observed in living pollen tubes. The vegetative nucleus is not observed in panels **F** to **J** but is stained with SYTO11. Bar in **J** = 20 μm for **A** to **J**

2004). It is exciting to speculate that the adaptation of this self-compatible plant, including a reduction in petal size and development of an automatic self-pollination system, occurred in such a short time period, i.e., within 320 000 years (Shimizu et al. 2004). An autogamous plant is useful for genetic analyses, but requires emasculation for artificial and cross pollination. In *Arabidopsis*, fertilization begins 5 h after pollination (Fauré et al. 2002). Each ovary contains about 50 ovules, which are fertilised by each pollen tube from the uppermost to the lowermost ovules (Hülskamp et al. 1995). The growth speed and germination rate of a pollen tube in vitro differ with the plant ecotype; pollen tubes of ecotype Columbia grow faster than those of Landsberg and Wassilewskij (WS).

Tobacco (*Nicotiana tabacum*) produces many pollen grains per anther. Pollen grains germinate with very high frequency (almost 100%) and grow at high rates of speed (~ 200 μm/h) to substantial lengths (over 1 cm; Read et al. 1992). In addition, transient expression in tobacco pollen can easily be induced by bombardment (e.g., Twell et al. 1989; Chen et al. 2002) and stable transformation can be induced using agrobacteria (e.g., Horsch et al. 1985). Thus, tobacco is commonly used for cytological analyses of pollen tube

growth in vitro. Tobacco plants are amphidiploid. The pollen is bicellular, i.e., it consists of one vegetative cell and one generative cell, and shows no self-incompatibility. *Nicotiana alata* has also been used for the study of gametophytic self-incompatibility, including identification S-RNase as a female sporophytic determinant (McClure et al. 1989; Barend et al., this volume). In tobacco, fertilization begins about 40 h after pollination.

Tomato (*Lycopersicon esculentum*) is a commercially important plant that belongs to the family Solanaceae, which also includes tobacco. Genetically modified organisms (GMO) were first commercialised using tomato (flavor saver) in 1994, and a tomato genome project (International Solanaceae Genome Project) launched in 2003. The most commonly used strong promoter for pollen vegetative cells, the *LAT52* promoter, was found in tomato. The pollen protein LAT52 interacts with a receptor-like kinase LePRK2 in what might be an autocrine signaling system (Tang et al. 2002). Tomato is autogamous; the pollen is bicellular (Fig. 1) and shows no self-incompatibility. Fertilization begins about 48 h after pollination.

Lily (*Lilium longiflorum*) has an extraordinarily large genome size compared to *Arabidopsis*. It has been estimated at 34 496 Mbp, i.e., about 300 times that of *Arabidopsis*. Lily cells are also large, reflecting the large genome size (Fig. 1), and, like tobacco, lily produces many pollen grains per anther; therefore, lily is useful for cytological, physiological and biochemical studies of pollen development and pollen tube growth (Hepler et al., this volume). Traditionally, the behaviour of chromosomes during meiosis has been studied in lily (Stern 1985). The isolation of a large number of generative cells at the same developmental stage is also possible, and this has led to the identification of generative-cell-specific proteins and genes (e.g., Ueda and Tanaka 1994; Mori et al. 2003). Additionally, molecules relating to pollen–pistil interactions have been found in lily, using in vitro systems (Kim et al. 2004; Johnson and Lord, this volume). Lily has bicellular pollen, which shows gametophytic self-incompatibility. Fertilization begins about 60 h after pollination occurs by passing through the style tissue, which reaches a length of 10 cm. *Agapanthus umbellatus*, *Hemerocallis* spp., *Ornithogalum* spp., *Gasteria verrucosa* and other *Lilium* spp. all belong to the Liliaceae (although *Gasteria* has also been placed in the Aloaceae) and have often been used to study pollen tube growth (Hepler et al., this volume).

Rice (*Oryza sativa*), one of the most important crops, is the most-consumed crop in the world. The entire genome sequence was the second whole plant genome to be published (International Rice Genome Sequencing Project 2005). Rice pollen is tricellular (Fig. 1). Generally, tricellular pollen is difficult to germinate in vitro (Brewbaker 1967), but pollen tube culture is possible (Kariya 1989; Khatun and Flowers 1995) provided that pollen grains are collected just at flowering (Khatun and Flowers 1995). Thus, rice pollen is rarely used for in vitro studies of pollen tube growth. However, studies of meiosis and anther and ovule development (Itoh et al. 2005) and

genome-wide quantitative trait loci (QTL) analyses of reproductive barriers (Harushima et al. 2001, 2002) have been progressing well. Fertilization begins 30 min after pollination.

Maize (*Zea mays*) another crop with many useful genetic resources available, is tricellular but pollen tube culture is possible (Walden 1993). Maize is suitable for the isolation of sperm cells and female gametophytic cells. Using isolation techniques, in vitro fertilization studies and transcriptome analyses have progressed in maize (Engel et al. 2003; Raghavan 2003; Weterings and Russell 2004). *ZmEA1*, which was found in the cDNA library of the egg cell, is also expressed in the synergid cell and contributes to pollen tube guidance to the female gametophyte (Márton et al. 2005). Fertilization begins 12 h after pollination (Mòl et al. 1994).

Brassica spp. (e.g., *B. campestris* (syn. *rapa*); *B. oleracea*; *B. napus*) and corn poppy (*Papaver rhoeas*) have both been used for studies of self-incompatibility, as have other self-incompatible plants including *Nicotiana alata* (Barend et al., this volume). Sporophytic self-incompatible plants include *Brassica* spp. (Brassicaceae) and sugar potato (*Ipomoea batatas*; Convolvulaceae). In *Brassica*, the female factor was identified as a S-locus receptor kinase, SRK, and the male factor was identified as a small, S-locus cysteine-rich (SCR) basic protein (SP11), which is the ligand of SRK (Takayama and Isogai 2003; Barend et al., this volume). Gametophytic self-incompatible plants include *Nicotiana alata* (Solanaceae), corn poppy (Papaveraceae), *Prunus dulcis* and *Pyrus pyrifolia* (almond and Japanese pear; Rosaceae), *Secale cereale* (rye; Poaceae) and *Antirrhinum* (Scrophulariaceae). In most of them, except for corn poppy and rye, the female factor was identified as S-RNase and the male factor was identified as an S haplotype-specific F-box (SFB) protein, which is related to protein degradation (Kao and Tsukamoto 2004). The pollen of *Brassica* spp. is tricellular, and pollen tube culture is possible (Sect. 2). The pollen of corn poppy is bicellular, and in vitro systems have been used in the study of self-incompatibility (Rudd and Franklin-Tong 2003).

Torenia fournieri possesses a naked embryo sac that protrudes from the micropyle of the ovule. Pollen tube attraction to the embryo sac can be directly observed in this plant (Higashiyama et al. 1998). Laser ablation experiments showed that the source of the attractant is the synergid cell (Higashiyama et al. 2001). The pollen of *Torenia* is bicellular, and the pollen tube grows well in vitro (Fig. 1). Transformation of *Torenia* has been performed for molecular breeding, including modification of flower colour (e.g. Aida et al. 2000). As an experimental plant, *Torenia* has many suitable characteristics for the study of plant reproduction, including the production of large numbers of flowers; relatively short generation time ($\sim$ 3 months from seeds to seeds); low plant height ($\sim$ 40 cm), allowing cultivation in chambers; allogamous reproduction without self-incompatibility, allowing easy control of pollination; and accessibility of the fertilization processes, allowing the observation of the

entire fertilization process (the precise time course of fertilization has also been determined; Higashiyama et al. 1997). Fertilization in *Torenia* begins 9 h after pollination. *Torenia* belongs to the Scrophulariaceae, as do snapdragon and *Mimulus*.

Snapdragon (*Antirrhinum majus*) is a commonly used experimental plant for developmental studies using transposable elements. The pollen of snapdragon is bicellular and the pollen tube grows well in vitro. Mascarenhas and Machlis (1962a) first showed that pollen tubes grow toward calcium ions in an in vitro assay using snapdragon (Johnson and Lord, this volume). The pollen does not show self-incompatibility, although some self-incompatible lines of *Antirrhinum* have been used for the study of gametophytic self-incompatibility (Barend et al., this volume). Fertilization begins about 48 h after pollination (Negre et al. 2003).

Cytoplasmic inheritance (Table 2) is not directly involved in pollen tube growth, but is important when considering the function of pollen in transmitting the paternal genome(s). Maternal, paternal, and biparental inheritance are known in the inheritance of both mitochondria and chloroplasts (plastids) in plants, which differs from the strict maternal inheritance that occurs in animals (Birky 1995). The majority of flowering plants, including biological models listed in Table 2, exhibit maternal inheritance. The inheritance of mitochondria is controlled independently from that of chloroplasts. For example, chloroplasts and mitochondria of *Medicago sativa* (alfalfa) are inherited biparentally and maternally, respectively (Forsthoefel et al. 1992), whereas those of *Musa acuminata* (banana) are inherited maternally and paternally, respectively (Fauré et al. 1994). These manners of cytoplasmic inheritance correlate well with the degradation or amplification of DNA in each organelle of the generative cell (Nagata et al. 1999); in the generative cells of *Medicago sativa*, chloroplast DNA is amplified while mitochondrial DNA is degraded, whereas in *Musa acuminata*, chloroplast DNA is degraded while mitochondrial DNA is amplified.

Plumbago zeylanica and some flowering plants show distinctive characteristics in the distribution of organelle DNAs in the generative and sperm cells (reviewed by Weterings and Russell 2004); these characteristics probably contribute to cytoplasmic inheritance. In *Plumbago zeylanica*, the sperm cell associated with the vegetative nucleus does not contain chloroplasts, but the other sperm cell, which is not associated with the vegetative nucleus, does (Russell 1984). These sperm cells contain amplified DNA (Sodmergen et al. 1995). The former sperm cell preferentially fertilises the central cell, and the latter fertilises the egg cell (Russell 1985). *Plumbago* also has a distinctive female gametophyte, which does not contain a synergid cell, although the base of the egg cell shows characteristic cell walls that resemble the filiform apparatus of the synergid cell (Russell 1985).

In addition to the common biological models mentioned above, there are other existing or developing biological models used in the study of pollen

Table 2 Fundamental characteristics of biological models used in the study of pollen tube growth

	Arabidopsis thaliana	*Brassica campestris*	*Nicotiana tabacum*	*Lycopersicon esculentum*	*Lilium longiflorum*	*Oryza sativa*	*Zea mays*	*Papaver rhoeas*	*Torenia fournieri*	*Antirrhinum majus*
Genome size (Mbp)[1]	125	564	5733	1005	34 496	389–466	2671	2573	—[2]	1568
Chromosome number (*n*)	5	9	24	12	12	12	10	7	9	8
Pollen cell number	Tricellular	Tricellular	Bicellular	Bicellular	Bicellular	Tricellular	Tricellular	Bicellular	Bicellular	Bicellular
Cytoplasmic inheritance	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal
Stigma type	Dry	Dry	Wet, lipid-rich	Wet	Wet carbo-hydrate-rich	Dry	Dry	Dry	Dry	Dry
Self-incompatibility	None	Sporophytic	None	None	Gameto-phytic	None	None	Gameto-phytic	None	None
Pollination (in a chamber)	Automatic	—	Automatic	Automatic	—	Automatic	Automatic[3]	—	None[4]	Automatic
Style type	Solid	Solid	Solid	Solid	Hollow	Solid	Solid	Solid[5]	Hollow	Solid
Pollen tube culture	Possible	Possible	Easy	Easy	Easy	Possible	Possible	Easy	Easy	Easy

[1] C-values (http://www.rbgkew.org.uk/cval/homepage.html) except for *Arabidopsis* (*Arabidopsis* Genome Initiative 2000) and rice (Yu et al. 2002; Goff et al. 2002; International Rice Genome Sequencing Project 2005). C-values of *Arabidopsis* and rice are 157 Mbp and 490 Mbp, respectively.
[2] We estimate the genome size of *Torenia* as several hundreds Mbp, judging from the fluorescence intensity of the generative cell nucleus stained with DAPI as shown in Fig. 1.
[3] The frequency of self-pollination is low, because female and male flowers bloom at different positions and times.
[4] Self-pollination never occurs, because of enough stigma-anther separation.
[5] A typical style is absent.

tube growth. For example, genome projects have been launched for *Medicago truncatula* and *Lotus japonicus* (Fabaceae). Interestingly, a relationship between the molecular mechanisms of pollen tube growth and infection thread formation in *L. japonicus* has been implied (Tansengco et al. 2004). *Pisum sativum* (pea), also in the family Fabaceae, is sometimes used for pollen tube microinjection (e.g., Li et al. 1999). *Impatiens*, *Portulaca*, *Camellia*, *Tradescantia* (e.g., spiderwort) and *Gibasis pellucida* (bridal veil) in the Commelinaceae, and *Trifolium* (e.g., clover), *Lathyrus odoratus* (sweet pea), *Melilotus* (e.g., sweet clover) in the Fabaceae, are often used in the classroom because of rapid germination and/or high germination frequency (these plants, except *Portulaca,* all possess bicellular pollen). The pollen of *Impatiens balsamina* (balsam) germinates very quickly (in 1–3 min) and at a high frequency in vitro. The pollen of *Portulaca* also shows a high germination frequency in vitro and is useful for the observation of pollen tubes on the papillar cell of the stigma. The pollen of *Camellia japonica* can germinate at a high rate in vitro, even at cold temperatures ($\sim 5\,^{\circ}C$), although it requires 30 min to several hours to germinate. Various additional plant species have been used for the observation of pollen tubes in the pistil in histochemical, ultrastructural and cytological studies (reviewed by Van Went and Willemse 1984).

In some species of flowering plants, the pollen requires an extraordinarily long time to pass from pollination to fertilization, similar to that of gymnosperms. In *Alnus* (alders), pollination to fertilization takes 2 months; intermittent pollen tube growth occurs, with a clear correlation between pollen tube growth and the developmental stages of the ovule and embryo sac (Sogo and Tobe 2005). These distinctive plant species may provide an opportunity to discover novel, but universal, mechanisms of pollen tube growth.

2
Pollen Tube Growth in Vitro

Upon pollination, the pollen germinates and the pollen tube grows toward the ovule, directed by complex intercellular communication with the pistil. Because the pollen tube growing through the pistil tissue is inaccessible, in vitro systems are used in various biological models. Pollen germination and pollen tube growth can be mimicked in vitro using chemically defined culture media under strictly controlled conditions. In this section, we compare characteristics and conditions of in vitro pollen tube growth in biological models.

2.1
Pollen Type

There are two types of pollen with regard to cell number, i.e., bicellular and tricellular pollen. Pollen mitosis II (division of the generative cell) of bicellu-

lar pollen occurs in the growing pollen tube, whereas that of tricellular pollen occurs in the maturing pollen grain (Fig. 1). The type of pollen is often correlated with the in vitro germination frequency and growth rate; generally, bicellular pollen is more easily cultivated in vitro. As shown in Table 2, bicellular pollen includes that of tobacco, tomato, lily, corn poppy, *Torenia* and snapdragon, and their pollen tubes grow well in vitro. In contrast, *Arabidopsis*, *Brassica* spp., rice and maize have tricellular pollen, and their pollen tubes are relatively difficult to cultivate. Brewbaker (1967) tested about 2000 species of flowering plants and showed that approximately 70% have bicellular pollen and 30% have tricellular pollen. Tricellular pollen was proposed to be a more evolved trait adapted to humid climates; interestingly, all aquatic species with submersed flowers shed pollen in a tricellular stage. Most of these genera are monotypic with respect to cell number, except for *Burmannia*, *Calliandra*, *Comanthosphace*, *Drosera*, *Euphorbia*, *Hymenocrater*, *Ipomoea*, *Lobelia*, *Plantago* and *Ruta*. The genes regulating the timing of division of the generative cell are still unknown, although water uptake may trigger the division of the generative cell in bicellular pollen.

2.2
Biological Models for the Study of Pollen Tube Growth in Vitro

The pollen of *Arabidopsis* and plants of the Solanaceae, including tobacco; Liliaceae, including lily and *Agapanthus*; Scrophulariaceae, including *Torenia*, and corn poppy are often used to study in vitro pollen tube growth. Among these plants, *Nicotiana alata*, corn poppy and self-incompatible *Antirrhinum* lines have been used to study self-incompatibility, and *Torenia* has been used to study pollen tube guidance to the embryo sac. The pollen of *Impatiens*, *Portulaca*, *Camellia*, the Commelinaceae, including spiderwort, and Fabaceae, including clover, is also often used for in vitro studies because of its rapid germination, high germination frequency, length of the pollen tube, and convenience in obtaining flower materials.

2.3
Culture Media and Conditions for Pollen Tube Growth in Vitro

To date, various culture media for in vitro pollen tube culture have been developed; some of these are summarised in Table 3. The medium of Brewbaker and Kwack (1963) is one of the most popular for pollen tube culture. Brewbaker and Kwack (1963) tested the pollen of several hundred species, particularly that of petunia and *Ornithogalum virens* (Liliaceae), to determine a basal medium (Table 3). The media of Hodgkin (1983), Jahnen et al. (1989) and Read et al. (1993) are also popular and have been used as bases from which to develop novel media for biological models. Variations in the media used for *Arabidopsis* pollen still exist, but the media used for lily

Table 3 Various media used for pollen tube culture*1

	petunia and *Ornithogalum* (Liliaceae)	*Arabidopsis*			tobacco	
Original paper	Brewbaker and Kwack 1963	Hodgkin 1983*2	Li et al. 1999	Derksen et al. 2002	Read et al. 1993	Chen et al. 2002
NH_4NO_3	—	—	—	—	—	—
HNO_3	—	—	—	—	—	100
KNO_3	100	100 (0.99 mM)	—	—	—	—
KCl	—	—	—	—	75 (1.0 mM)	—
$Ca(NO_3)_2 \cdot 4H_2O$	300	600 (2.54 mM $Ca(NO_3)_2$)	236 (1 mM)	—	—	—
$CaCl_2$	—	—	111 (1 mM)	528 (700; $CaCl_2 \cdot 2H_2O$)	111 (1.0 mM)	700
$MgSO_4 \cdot 7H_2O$	200	217 (0.88 mM)	—	—	96 (0.8 mM $MgSO_4$)	409 (200; $MgSO_4$)
$Mg(NO_3)_2 \cdot 6H_2O$	—	—	—	—	—	—
$MgCl_2$	—	—	—	—	—	—
KH_2PO_4	—	—	—	—	—	—
$K_3PO_4 \cdot H_2O$	—	—	—	—	—	—
$MnSO_4 \cdot 4H_2O$	—	—	169 (1 mM)	—	—	—
$ZnSO_4 \cdot 7H_2O$	—	—	—	—	—	—
H_3BO_3	100	100 (1.62 mM)	100	100	99.2 (1.6 mM)	100
$CuSO_4 \cdot 5H_2O$	—	—	—	—	(30 μM $CuSO_4$)	—
$Na_2MoO_4 \cdot 2H_2O$	—	—	—	—	—	—
Sucrose	10%	20% (585 mM)	18%	20%	5%	2%
Polyethylene-glycol (MW)	—	—	—	3% (4000)	12.5% (6000)	15% (3350)
Casein	—	—	—	—	300	—
MES	—	—	—	—	15 mM	20 mM
TAPS	—	20 mM	—	—	—	—
pH	—	8 (NaOH)	7	—	5.9 (KOH)	6
Agarose	—	0.5%	—	0.7% (Bacto-Agar)	—	—
Rifampicin	—	—	—	—	10	—

pollen have been improved over many years and have almost converged (Table 3).

At least three substances, plus water, are required in the pollen tube culture medium. The first substance is calcium, which is necessary for pollen tip growth (see Hepler et al., this volume). Calcium is usually added to the medium at 10–600 mg/L as nitrate or chloride salts. The second substance is borate, which may be necessary for cell wall formation (Loomis and Durst 1992). Borate is usually added as boric acid, and a concentration of 100 mg/L

Table 3 (continued)

	Nicotiana alata				*Agapanthus*	corn poppy	*Torenia*
Original paper	Jahnen et al. 1989	Holdaway-Clarke et al. 2003[*3]	Kim et al. 2003	Prado et al. 2004	Malhó and Trewavas 1996	Franklin-Tong et al. 1988	Higashi-yama et al. 1998[*4]
NH_4NO_3	—	—	—	—	—	—	80
HNO_3	—	—	—	—	—	—	—
KNO_3	100	—	100 (0.99 mM)	—	—	—	125
KCl	—	7.5 (0.1 mM)	—	75 (1.0 mM)	200	—	—
$Ca(NO_3)_2 \cdot 4H_2O$	700	—	300 (1.27 mM)	—	—	—	500
$CaCl_2$	—	11.1 (0.1 mM)	—	55.5 (0.5 mM)	200	272 (360; $CaCl_2 \cdot 2H_2O$)	—
$MgSO_4 \cdot 7H_2O$	200	—	—	—	—	—	125
$Mg(NO_3)_2 \cdot 6H_2O$	—	—	—	—	—	100	—
$MgCl_2$	—	—	—	—	200	—	—
KH_2PO_4	—	—	—	—	—	—	125
$K_3PO_4 \cdot H_2O$	—	—	—	—	—	100	—
$MnSO_4 \cdot 4H_2O$	—	—	—	—	—	—	3
$ZnSO_4 \cdot 7H_2O$	—	—	—	—	—	—	0.5
H_3BO_3	100	99.2 (1.6 mM)	10 (0.162 mM)	99.2 (1.6 mM)	100	100	10
$CuSO_4 \cdot 5H_2O$	—	—	—	—	—	—	0.025
$Na_2MoO_4 \cdot 2H_2O$	—	—	—	—	—	—	0.025
Sucrose	2%	7%	10%	6%	2.5%	12%	1%
Polyethylene glycol (MW)	15% (4000)	—	—		—	—	13% (4000)
Casein	—	—	—	—	—	—	500
MES	—	15 mM	—	0.05 mM	—	—	—
TAPS	—	—	—	—	—	—	—
pH	—	6 (KOH)	5.2	6.0	6.0	—	5.8 (without adjustment)
Agarose	—	—	1.0%	—	—	0.4%	1.5%[*5]
Rifampicin	—	—	—	—	—	—	—

[*1] Units are mg/L unless otherwise noted.
[*2] Originally established for pollen tube culture in *Brassica oleracea* but often used for pollen tube culture in *Arabidopsis*.
[*3] Established for pollen tube culture in *Lilium formosanum*.
[*4] The 4% sucrose is now replaced by 13% PEG 4000 to improve the fertilization frequency as described in Sect. 13.2.
[*5] Ultra-low gelling temperature agarose.

appears sufficient for most flowering plants. The third substance is sucrose, which is necessary to adjust the osmotic pressure and may be used as a carbon source for respiration and the synthesis of starch, lipids, amino acids

and nucleic acids (Vasil 1987). Sucrose is the most suitable sugar source in most flowering plants (Vasil 1987). The optimum osmotic pressure differs among biological models; for example, the optimum sucrose concentration is 10–20% for *Arabidopsis* (Hodgkin 1983; Li et al. 1999; Derksen et al. 2002; Schreiber and Dresselhaus 2003), 9–18% for tobacco (Cheung et al. 2002; Romagnoli et al. 2003), 5–10% for lily (Vidali et al. 2001; Holdaway-Clarke et al. 2003; Kim et al. 2003; Prado et al. 2004), 2.5% for *Agapanthus* (Malhó and Trewavas 1996), 11% for maize (Walden 1993), 20% for rice (Kariya 1989), 12% for corn poppy (Franklin-Tong et al. 1988) and 5% for *Torenia* (Higashiyama et al. 1998).

In addition, other inorganic ions (e.g., K^+, Mg^{2+}; Brewbaker and Kwack 1963), buffers to control pH (e.g., 2-Morpholinoethanesulfonic acid, monohydrate (MES); Tupy and Rihova 1984) and organic compounds (e.g., amino acids, casein hydrolysate; reviewed by Vasil 1987) are used in the media to promote pollen tube germination and growth, depending on the plant species. The frequency and timing of the generative-nucleus division also depended on the chemical composition of the medium in tobacco (Read et al. 1993). It is noteworthy that polyethyleneglycol (PEG) 4000–8000 has a dramatic effect on pollen tube germination and growth (Zhang and Croes 1982; Jahnen 1989; Read et al. 1993; Barinova et al. 2002), although the physiological action of PEG is unknown. PEG has been widely used in pollen tube culture media for *Arabidopsis*, tobacco, *Torenia* and snapdragon (Barinova et al. 2002; Schreiber and Dresselhaus 2003; Table 3). In *Torenia*, PEG 4000 increased the viability of both the pollen tube and the naked embryo sac, whereby the frequency of pollen tube attraction in vitro increased fourfold (Higashiyama et al. 2000). However, high concentrations of PEG tend to precipitate in solid media. Thus PEG is sometimes replaced by sucrose when used in solid media (e.g., Cheung et al. 2002). Osmotic potential of 15% PEG 4000 (327 mOsm/kg) corresponds to that of 9% sucrose (325 mOsm/kg) (Jahnen et al. 1989). In the medium for *Torenia*, 13% PEG 4000 in 1.5% ultra-low gelling temperature agarose is the maximum concentration that balances the effect of PEG 4000 and the extent of precipitation (Table 3). As in cell fusion experiments, PEG appears to remain effective for only one month after its dissolution in water, and its effect is also lost by autoclaving.

The effectiveness of simple media should be tested first. Environmental conditions, such as humidity and aeration, and physiological conditions of the flower are critical and can be tested using a simple medium. Pollen germination may also depend on the population effect, i.e., the pollen concentration. Phytosulphokine (PSK), a peptide hormone of flowering plants, is an intercellular signalling molecule (Chen et al. 2000). It should also be noted that Na^+ strongly inhibits pollen tube growth in most flowering plants. Thus, KOH, rather than NaOH, is recommended to adjust the pH. The optimal temperature for pollen tube culture is usually 20–30 °C, and the growth rate of pollen tubes depends on the temperature.

The medium for the in vitro *Torenia* system (Higashiyama et al. 1998, 2001) is described in Table 3. *Torenia* pollen tubes grow well in a simple medium containing 300 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 100 mg/L H_3BO_3, 1% sucrose and 13% PEG 4000 (Fig. 1), but this medium does not support the culture of ovules that have a naked embryo sac. Therefore, Nitsch's medium (1951), originally established for the culture of excised ovaries, was modified for the cultivation of both the pollen tube and ovule together.

2.4 Semi-in Vitro Pollen Tube Growth

A pollen tube can grow autonomously to some extent using its own nutrients; however, the resultant pollen tube is much shorter than the style. Pollen tubes perceive many molecules in the extra-cellular matrix (ECM) of the pistil, and no medium can produce pollen tube growth similar to that which occurs in the pistil. Thus, a semi-in vitro (also called semi-in vivo) system is sometimes used for pollen tube culture, wherein pollen tubes grow through a cut style. Pollen tubes germinate on the stigma, grow through the cut style, and enter the culture medium from the cut end of the style. Pollen tubes grown semi-in vitro show a higher growth rate and more normal morphology than those germinated on artificial medium. For example, in *Torenia* at 25 °C, pollen tubes grow at 2.3 mm/h in the pistil (in vivo), 0.6 mm/h in medium after germinating on the medium (in vitro), and 1.2 mm/h in the same medium after germinating on the stigma and passing through the style (semi-in vitro). Moreover, semi-in vitro growth is necessary for the capacitation-like mechanism of the pollen tube to respond to attractant from the synergid cell in *Torenia* (Higashiyama et al. 1998). Similar phenomena have been observed in lily (Janson 1993) and *Aechmea fasciata* (Bromeliaceae; Vervaeke et al. 2003); pollen tubes grown semi-in vitro more frequently penetrate the micropyle of the ovule than those grown in vitro. Among the biological models, semi-in vitro pollen tube growth has also been used in tobacco (Cheung et al. 1995), as described in (Johnson and Lord, this volume).

For semi-in vitro pollen tube growth, the medium must be prepared as carefully as that for in vitro pollen tube culture. It is important that the style tissue, including the cut end, is well-maintained on the medium so that pollen tube growth inside the tissue is supported. Any self-incompatibility of the plant species should also be noted before semi-in vitro culture is performed.

3 Study of Pollen Tube Guidance in Vitro

During growth in the pistil, the pollen tube perceives multi-step directional controls from the pistil (see Johnson and Lord, this volume). To study the

mechanisms of pollen tube guidance and to identify guidance cues, many in vitro systems have been developed using various biological models. In this section, we compare such in vitro systems to illustrate how the characteristics of each biological model have been used to study pollen tube guidance.

3.1 In Vitro Systems to Identify Candidate Chemoattractants

Many in vitro systems have been developed to evaluate pollen tube chemotropism; some of these are summarised in Fig. 2. According to Mascarenhas and Machlis (1962b), Van Tieghem (1869) was the first to suggest the possibility of chemo-attraction of the pollen tube in vitro. He cultivated ovules with pollen tubes for some species of flowering plants and observed that the pollen tube grew toward the ovule. Since then, many plant biologists have attempted to identify the guidance cue that navigates the directional growth of the pollen tube with various in vitro tests, using tissues of the pistil (Mascarenhas and Machlis 1962b). Biochemical properties, such as heat-stability and molecular size, were examined in various plants using these in vitro systems until the 1960s. These attempts led to the screening of inorganic ions. Mascarenhas and Machlis (1962a) identified calcium ion derived from the pistil tissue as a candidate chemoattractant of the pollen tube using an in vitro system in snapdragon, as shown in Fig. 2A. Snapdragon was chosen because it consistently showed chemotropic responses in various in vitro sys-

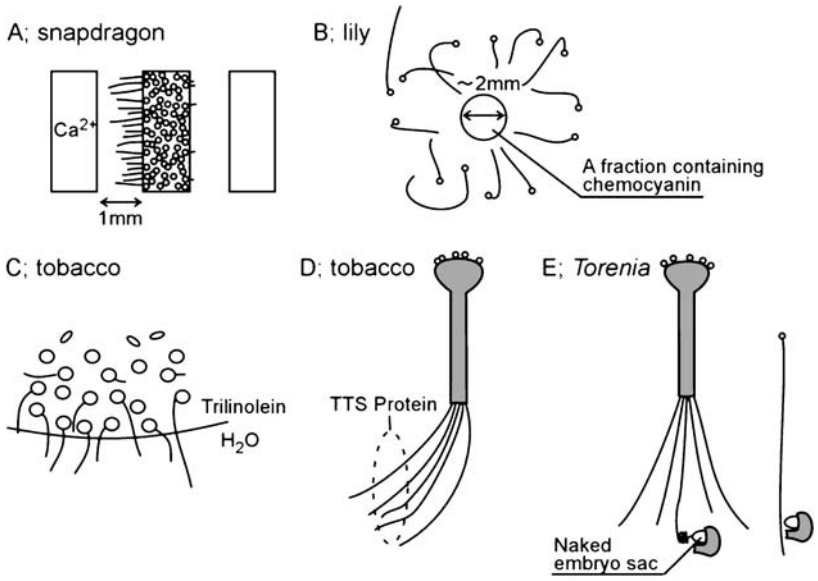

Fig. 2 Various in vitro systems used to assay pollen tube chemotropism

tems and because large numbers of flowers could be obtained throughout the year (Mascarenhas and Machlis 1962c).

A number of histochemical analyses have supported these results by showing the existence of abundant calcium ion along the path of the pollen tube, especially in the synergid cell (reviewed by Higashiyama 2002). However, it was difficult to distinguish between guidance and growth stimulation using classical in vitro tests. In addition, pollen tubes tend to grow parallel when pollen grains are placed in a row. There was no evidence that calcium ion was the true attractant in the pistil; therefore, the existence of a pollen tube attractant has been a confounded issue. For example, Heslop-Harrison and Heslop-Harrison (1986) discussed the existence of the attractant in their review entitled "Pollen-tube chemotropism: fact or delusion?" Kim et al. (2004) finally identified a plantacyanin (blue-copper cell wall protein), a 9.9-kDa basic protein named chemocyanin, in the stigma extract of lily (Johnson and Lord, this volume). The function of plantacyanins in plant cell wall is unknown, but many are capable of redox reactions. In their assays, chemocyanin showed chemoattraction of the pollen tube (e.g., Fig. 2B). Lily is one of the species that shows typical chemotropic responses in vitro to pistil tissues, including the stigma and the ovule (Miki 1954; Welk et al. 1965). Because of the large pollen size, it is also possible to place individual pollen grains in a row to clearly observe the behaviour of each pollen tube (Fig. 2B).

Two types of stigma are recognised: wet and dry (Heslop-Harrison and Shivanna 1977; Table 2). The stigma of lily is wet, with a carbohydrate-rich exudate. In contrast, the stigmas of tobacco and petunia are wet, but lipid-rich. In other plants, such as *Arabidopsis*, *Brassica* spp., rice, maize, corn poppy, *Torenia* and snapdragon, the stigma is dry. The lipid fraction of the stigma exudate contains many triacylglycerides; one of these, trilinolein (unsaturated triacylglyceride), allows pollen tubes to penetrate stigmas that have had genetic ablation of the secretory zone and cannot produce exudate. In an in vitro assay, pollen grains placed in trilinolein germinated in the vicinity of water solidified with 0.7% agarose, and some pollen tubes nearest to the trilinolein–water boundary grew toward the water (Fig. 2C; Wolters-Arts et al. 1998). Few pollen grains placed in other saturated triacylglycerides germinated, and those that did showed no directional growth. A difference in water supply was also evident when different lipids were used. Thus, adequate water supply, depending on physicochemical properties of the lipids, seems necessary for pollen tube germination and directional growth (Wolters-Arts et al. 1998). Similar results were obtained using an in vitro system of *Nicotiana alata*; these results supported the conclusion that the gradient of water should be the guidance cue (Lush et al. 1998). The *Arabidopsis* mutant *pollen–pistil interaction (pop)1* is defective in the synthesis of long-chain lipids, and its pollen does not become hydrated on the stigma because of an impaired pollen coat (Preuss et al. 1993). Application of trilinolein to the stigma enabled *pop1*

pollen grains to produce tubes that penetrated the stigma (Wolters-Arts et al. 1998). Thus, trilinolein and an adequate water gradient are likely to play essential roles, even in dry stigmas.

The style is the tissue that connects the stigma and the ovary. The length of the style differs among plant species: for example, the style measures ~ 100 μm in *Arabidopsis*, ~ 2 mm in rice, ~ 2 cm in *Torenia*, ~ 4 cm in tobacco, ~ 10 cm in lily and ~ 30 cm in maize. Two main types of style are recognised, i.e., "hollow" (open; e.g., lily and *Torenia*) and "solid" (filled with transmitting tissue; e.g., *Arabidopsis*, *Brassica* spp., tobacco, tomato, petunia, rice, maize, corn poppy and snapdragon). In the style, pollen tubes generally grow straight toward the ovary at high growth rates. Cheung et al. (1995) used an in vitro system to test whether transmitting-tissue-specific (TTS) proteins of tobacco have the ability to attract the pollen tube (Fig. 2D). TTS proteins are arabinogalactan proteins (AGPs) in the ECM of the transmitting tissue (style) that are incorporated into the cell wall of the pollen tube and promote tube growth. The authors used pollen tubes growing semi-in vitro to examine changes in the direction of growth of the pollen tube, and observed that pollen tubes turned toward the medium containing TTS proteins (Fig. 2D). It has been argued whether this behaviour of pollen tubes actually indicates the ability of TTS proteins to attract the pollen tube, because it appeared difficult to distinguish between growth promotion and attraction in this system (Lush 1999). However, growth promotion simply cannot account for the directional change of the pollen tube. Other molecules that promote pollen tube growth, such as sucrose, do not show the same effect (Cheung et al. 1995). Interestingly, Mascarenhas and Machlis (1962a) similarly observed that the effect of calcium ion was apparently different from that of sucrose and yeast extract. In contrast, Lush (1999) pointed out that the pollen tubes in the in vitro system of Cheung et al. (1995) did not show trapped behaviour at the point where the concentration of TTS proteins was maximal. In fact, such a trapped behaviour has not been demonstrated well in vitro, except for the in vitro *Torenia* system, as described below. In the developing nervous system, milestones that emit the attractant protein, netrin, exist along the path of the growth cone. The high concentration of netrin renders the growth cone non-sensitive to netrin, causing the cone to approach the next milestone (Shirasaki et al. 1998). Although it is unknown whether the reproductive system has a similar mechanism, these factors make it difficult to demonstrate pollen tube attraction in vitro in a convincing manner.

In the ovary, pollen tubes change their behaviour from straight to meandering growth, climb up the funiculus of the ovule to enter the micropyle, and then grow toward the target female gametophyte, the embryo sac. The entrance of the micropyle seems the most plausible site for the release of a chemoattractant. In *Arabidopsis* mutants defective in embryo sac development, the embryo sac was shown to be necessary for directional pollen tube growth toward the funiculus and the micropyle (Hülskamp et al. 1995; Ray

et al. 1997; Shimizu and Okada 2000). However, the nature of the guidance cue remains unknown. Higashiyama et al. (1998) developed an in vitro system in *Torenia* whereby pollen tubes growing semi-in vitro through the cut style grew toward the micropylar end of the naked embryo sac in the medium, with no need to contact surrounding sporophytic tissues (Fig. 2E). In most cases, pollen tubes did not enter the embryo sac smoothly; rather, they continued to grow toward the micropylar end of the embryo sac, but slipped on the surface of the filiform apparatus of the synergid cell and formed narrow coils (Fig. 2E). Moreover, when an ovule that had attracted, but not received the pollen tube, was moved using a micromanipulator, the pollen tube was observed to trail the embryo sac (unpublished data). This pollen tube behaviour clearly indicates that some diffusible signal is derived from the micropylar end of the embryo sac.

Laser ablation experiments in the in vitro *Torenia* system have shown that the two synergid cells were the source of the attractant (Higashiyama et al. 2001). In *Arabidopsis*, a reverse genetic study of the synergid cell-specific MYB 98 transcription factor confirmed that the synergid cell governs pollen tube guidance at the entrance of the micropyle (Kasahara et al. 2005). Strong species specificity is observed in the pollen tube attractant, suggesting that the attractant may be some molecule, such as a peptide, synthesised in the synergid cell, rather than calcium ion or GABA (Palanivelu et al. 2003; Higashiyama et al. 2003). The attractant chemical is still unknown, although ZmEA1, which is expressed in the egg apparatus of maize, is one of the candidates. ZmEA1 is secreted near the micropyle, as observed using a green fluorescent protein (GFP) fused with ZmEA1, and governs pollen tube guidance at the micropyle, as observed in RNAi knockdown lines (Márton et al. 2005). Proteins similar to ZmEA1 exist only in monocots (McCormick and Yang 2005), and the ability of ZmEA1 to attract pollen tubes has not been shown.

3.2 Comparison of Biological Models with Regard to in Vivo Pollen Tube Guidance

The architecture of the pistil and ovary (including its size and the number of ovules) differs among biological models. As described above, there are different types of stigma and style, and their sizes also differ considerably. For example, there are several types of placenta (placentation) such as parietal (e.g., *Arabidopsis*, *Brassica* spp. and corn poppy), axile (e.g., tobacco, tomato, petunia, lily, *Torenia* and snapdragon) and basal (e.g., rice and maize). In *Arabidopsis*, a typical placenta is not observed, and the placental tissue is usually called a septum. The number of ovules in one ovary is $\sim$ 50 in *Arabidopsis*, $\sim$ 3000 in tobacco, $\sim$ 400 in lily, 1 in rice, and $\sim$ 500 in *Torenia*.

The size of the ovule may affect the number of steps involved in pollen tube guidance by the embryo sac because the effective distance of chemoattraction

is limited mathematically (Lush 1999). In the in vitro *Torenia* system and *Arabidopsis*, the effective distance of attraction by the synergid cell is only a few hundred micrometers at maximum (Higashiyama et al. 2003; Kasahara et al. 2005). In *Arabidopsis*, pollen tube guidance at the ovule is governed by the embryo sac, with at least two control steps (Shimizu and Okada 2000) but in larger ovules more steps may be necessary.

4 Perspectives

Here, we have compared the characteristics of biological models used in the study of pollen tube growth, with a special focus on in vitro culture. Use of these biological models will provide opportunities to identify novel genes, molecules and physiological mechanisms involved in pollen tube growth and check the universality and specificity of each finding. It is foreseen that a large amount of data collected in the near future will focus on *Arabidopsis*. However, the differences outlined in this chapter stress that extrapolations to other species must be critically examined using in vitro systems of other biological models.

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