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Review

How pollen tubes grow

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Abstract

Sexual reproduction of flowering plants depends on delivery of the sperm to the egg, which occurs through a long, polarized projection of a pollen cell, called the pollen tube. The pollen tube grows exclusively at its tip, and this growth is distinguished by very fast rates and reaches extended lengths. Thus, one of the most fascinating aspects of pollen biology is the question of how enough cell wall material is produced to accommodate such rapid extension of pollen tube, and how the cell wall deposition and structure are regulated to allow for rapid changes in the direction of growth. This review discusses recent advances in our understanding of the mechanism of pollen tube growth, focusing on such basic cellular processes as control of cell shape and growth by a network of cell wall-modifying enzymes, molecular motor-mediated vesicular transport, and intracellular signaling by localized gradients of second messengers.

Keywords: Pollen tube growth; Pectin methylesterases; Molecular motors; Rab and Rop/Rac GTPases

Introduction: pollen and pollen tube

Sexual reproduction of flowering plants requires delivery of the sperm to the egg. The process begins with deposition of the pollen grain, containing the male gametes and delivered by an insect, wind or other means, on the female stigmatic tissue. If the pollen–stigma interaction is compatible, the pollen grain hydrates and germinates shortly following landing on the stigma. During germination, a defined area in the pollen plasma membrane – the tip growth domain to which post-Golgi vesicles are targeted and fused, promoting directional growth – is established, and the pollen tube elongation begins, often reaching astounding rates of growth. For example, the maize pollen tube can grow as fast as 1 cm/h and extend to about 1 ft in length within 24 h (Barnabas and Fridvalszky, 1984).

During the elongation process, the pollen cytoplasm, vegetative nucleus and sperm cells are transported within the tube, which grows through intercellular spaces in the pistil. To serve as a conduit through which the fertilizing sperm cells can travel, the tube plots its course through the transmitting tissues, eventually reaching the eggs within the ovary (Johnson and Preuss, 2002; Kim et al., 2003; Palanivelu and Preuss, 2000; Ray et al., 1997). Molecular cues, such as the transmitting tissue-specific (TTS) protein in tobacco (*Nicotiana tabacum*) (Cheung et al., 1995) or gamma-aminobutyric acid (GABA) in *Arabidopsis* (Palanivelu et al., 2003), are located within the pistil tissues and guide the tube to deliver the sperm cells to the embryo sac for fertilization (Cheung et al., 1995; Johnson and Preuss, 2002; Lord and Russell, 2002). Additionally, the female tissues provide pollen tube-attracting signals as has been shown using mutants with delayed development of the embryo sac, that fail to attract nascent pollen tubes (Shimizu and Okada, 2000).

Over decade ago, two major biochemical mechanisms driving the pollen tube elongation were discovered: a steep calcium gradient within the pollen tube tip and the contribution of actin microfilaments to the elongation process. Pollen tubes exhibit a sharp, tip-focused intracellular calcium gradient that drives and orients their apical growth. While plant cells generally maintain a cytosolic calcium concentration of approximately 100 nM, during pollen tube growth a much higher concentration of several micromolar calcium is

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found in the apical few micrometers of the growing tip (see Holdaway-Clarke and Hepler, 2003 for review; Malhó et al., 1994). Apical calcium concentration in the nascent lily (*Lilium longiflorum*) pollen tubes, for instance, is estimated at 3–5 μM, while the rest of the tube retains the normal cellular calcium levels (~100 nM). Interestingly, this steep apical gradient returns to the basal cellular level within ~20 μm from the tip apex (Miller et al., 1992; Obermeyer and Weisenseel, 1991; Pierson et al., 1994; Rathore et al., 1991). Several studies have shown, that calcium gradient dissipation by various methods, including BAPTA buffer injections, mild thermal shock, calcium channels blockers and others, lead to the inhibition of the pollen tube growth (Li et al., 1996; Pierson et al., 1994; Rathore et al., 1991). Also, artificial generation of a focused elevated internal calcium level through localized photolysis of caged calcium has altered tip growth directionality, again supporting the notion of the necessity of the calcium gradient for the pollen tube growth (Malhó and Trewavas, 1996). The gradient high-point is in the immediate vicinity of the tip apex, and it appears to be derived, at least to some extent, from the influx of extracellular calcium through stretch-responsive channels activated by deformations in the nascent tip wall (Holdaway-Clarke et al., 1997; Pierson et al., 1994, 1996). However, although calcium-permeable channels are likely to contribute to the gradient formation and control, the specific molecular mechanisms of this process remain largely unknown (Dutta and Robinson, 2004).

Actin is essential for the polarized tip growth (Geitmana et al., 2000; Vidali and Hepler, 2001; Vidali et al., 2001). Together with myosin motors, actin microfilaments support vesicular transport and other crucial processes critical for the tube growth, since inhibition of actin polymerization by drugs, such as latrunculin B and cytochalasin B, effectively blocks pollen tube elongation (Gibbon et al., 1999; Miller et al., 1999). Although the abundance of the longitudinal actin filaments within the pollen tube is beyond doubt, it remains unclear which specific form(s) or population(s) of the F-actin are directly involved in the tip growth.

The role of microtubules in pollen tube growth, on the other hand, has been quite puzzling, since in two species of the *Nicotiana* family, tobacco and *Nicotiana alata*, and in *Endymion nonscriptus*, pharmacological disruption of microtubules by oryzalin or colchicine had no effect on pollen tube elongation (Åström et al., 1995; Heslop-Harrison et al., 1988; Laitiainen et al., 2002). Conversely, in Norway spruce (*Picea abies*), oryzalin or colchicine treatments partially blocked pollen germination and growth, generating swollen tips and shorter tubes (Anderhag et al., 2000). Also, microtubule inhibitors colchicine and propham inhibited pollen tube growth in another *Nicotiana* species, *Nicotiana sylvestris* (Joos et al., 1994).

The functions of the actin microfilaments and their myosin motors and the roles of ionic gradients, mainly that of calcium, in the pollen tube growth, the mechanisms of the pollen chemotaxis to the ovule, and the identity and action of pistil-guiding signals have been the subjects of numerous excellent reviews (Bedinger, 1992; Feijo et al., 2001; Hepler et al., 2001; Higashiyama et al., 2003; Johnson and Preuss, 2002; Mascarenhas, 1993; McCormick and Yang, 2005; Palanivelu and Preuss, 2000; Taylor and Hepler, 1997). Conversely, less attention was paid to recent significant advances in our understanding of the roles of (i) the cell wall-modifying enzymes pectin methylesterases (PMEs) and cellulose synthases, (ii) small GTPases involved in the regulation of traffic of membrane vesicles and dynamics of actin microfilaments, (iii) microtubule-associated molecular motors dyneins and kinesins, and (iv) second messengers, such as the calcium/phospholipid system and cAMP. This review discusses our up-to-date knowledge of these aspects of the pollen tube growth.

Cell wall-modifying enzymes: PMEs and cellulose synthases

Pectin methylesterases (PMEs)

In most plant species, the pollen tube cell wall consists of two layers, the inner sheath of callose and outer coating containing mainly pectin with cellulose and hemicellulose. The pollen tube grows exclusively at its tip, where the newly synthesized cell wall is continually forming (Taylor and Hepler, 1997). A single pectin layer, lacking callose or cellulose, forms the tip cell wall (Ferguson et al., 1998) and provides this area with sufficient rigor to maintain the cellular integrity, on the one hand, and with plasticity to allow directional tube growth, on the other (Steer and Steer, 1989).

During the tube elongation process, pectins are polymerized, as well as methyl esterified and modified with side chains within the Golgi apparatus, and transported in the Golgi-derived vesicles to the pollen tip (Li et al., 1995; Staehelin and Moore, 1995; Sterling et al., 2001). Following vesicle discharge, homogalacturonan – a linear polymer composed of 1,4-α-D-galacturonic acid residues, which represent the major component of pectin – is gradually deesterified by pectin methylesterases (PMEs). The deesterification of the polygalacturonic acid chain converts the methoxyl groups into carboxyl groups, exposing the acidic residues which then are cross-linked by calcium ions, creating a new layer of pectin (Catoire et al., 1998). The key role of PMEs during cell wall formation requires tight regulation of this enzymatic activity, which is achieved most likely through the properties of the surrounding medium, with pH being one of the key parameters. Specifically, the localized reduction in pH, due to the deesterification process, may promote cell wall relaxation by stimulating the activity of several cell wall-loosening hydrolases, such as polygalacturonases and pectate lyases (Ren and Kermode, 2000; Wen et al., 1999). The interplay between these opposing effects – cell wall stiffening by the PME action and cross-linking by calcium versus cell wall loosening by different hydrolases – likely regulates the directional growth of the pollen tube tip (Bordenave, 1996; Catoire et al., 1998; Moustacas et al., 1991).

PMEs represent a large family of plant enzymes originally discovered and characterized in the kiwi fruit (Balestrieri et al., 1990; Camardella et al., 2000; Giovane et al., 1995). For

example, the *Arabidopsis* genome encodes over 60 proteins containing the PME catalytic domain, which is frequently accompanied by one or more PME inhibitor domains (PMEI) on the same polypeptide chain (Markovic and Jornvall, 1992; Tian et al., 2006). The PMEI domain, also known as the PME pro-region, is thought to regulate the enzymatic activity by intra-molecular inhibition the PME catalytic domain; interestingly, the pro-region is also involved in the cell wall targeting of PME but it does not act, as previously proposed, as a chaperone for the PME folding (Micheli, 2001). In addition, virtually all PMEs contain a pre-region that includes the signal peptide, which mediates protein targeting to the endoplasmic reticulum (ER) for subsequent secretion of PME into the cell wall (Bosch and Hepler, 2005; Dorokhov et al., 2006; Micheli, 2001). The members of the PME multigene family are well known to play a role in plant growth and development and in plant–pathogen interactions; they modulate pH and ion balance and affect cell wall porosity (Nairn et al., 1998 and references therein; Pressey, 1984), and have been implicated in plant responses to pathogen attack (Markovic and Jornvall, 1986) and in plant virus movement between host cells (Chen and Citovsky, 2003; Chen et al., 2000; Dorokhov et al., 1999).

The essential function of PMEs in the formation of plant cell walls also implies a major role for PME in the pollen tube growth, which obviously involves dramatic changes in the cell wall dynamics. Indeed, recent analysis of pollen-specific transcriptome of *Arabidopsis* indicated that numerous PMEs are specifically expressed in floral buds, including pollen (Pina et al., 2005). This aspect of the pollen tube growth, however, has been largely neglected; it has received attention only in the last 2 years, and the resulting studies have already yielded valuable insights into the complexity and importance of the PME involvement in pollen development (Bosch et al., 2005; Jiang et al., 2005; Pina et al., 2005; Tian et al., 2006).

The first functional analysis of a pollen tube-expressed PME focused on a novel *Arabidopsis* gene, *VANGUARD1* (*VGD1*) (Jiang et al., 2005). *VGD1*, which encodes a 595-amino-acid-long PME-like protein, is expressed specifically in the pollen grain and tube and localizes to the plasma membrane and the cell wall. Although the pollen grains of a pollen-specific gametophytic *vgd1* mutant of *Arabidopsis* germinated normally on the stigmatic surface and invaded the stigma, their tubes grew much slower than those of the wild-type pollen within the style and the transmitting tract (Jiang et al., 2005). In addition, the *vgd1* pollen tubes were unstable, bursting more frequently then the wild-type tubes when germinated and grown *in vitro*, indicating that the *vgd1* mutation alters the mechanical properties of the cell wall. While the *vgd1* mutant plants were defective in normal growth of pollen tubes in the style and transmitting tract tissues, and they showed a reduced male fertility, no other significant phenotypic alternations were detected, confirming the pollen-specific functionality of VGD1. Biochemical characterization of the homozygous *vgd1* mutant pollen (Jiang et al., 2005) revealed a small, but consistent reduction in the overall PME activity to 82% of the wild-type plants known to express more than 12 different PMEs in their pollen (Pina et al., 2005; Tian et al., 2006). These observations indicated that VGD1 is indeed a functional PME, and that even a relatively small alteration in the balance of the PME activities in pollen results in far-reaching developmental, functional, and morphological/mechanical changes in the pollen tube (Jiang et al., 2005).

The second pollen-specific PME characterized to date is the *Arabidopsis* AtPPME1 (Tian et al., 2006) identified by the Fluorescent Tagging of Full-Length Proteins (FTFLP) technique (Tian et al., 2006). FTFLP – which inserts an autofluorescent marker sequence, e.g., green fluorescent protein (GFP) or its various spectral variants, within the last exon of a full-length genomic sequence of the gene of interest controlled by its native regulatory elements – faithfully reproduces the native level and pattern of gene expression as well as the subcellular localization of the fluorescently tagged gene product (Tian et al., 2004). Characterization of expression patterns of AtPPME1 by FTFLP revealed that this protein is restricted solely to the pollen grains, accumulating around the cell periphery and in the cytoplasm and the ER/Golgi endomembrane system (Fig. 1A). Within the growing pollen tube, AtPPME1, similarly to VGD1 (Jiang et al., 2005), was dispersed throughout the entire volume of the tube (Tian et al., 2006) (Figs. 1B, C). Reverse genetics experiments using a pollen-specific gametophytic *atppme1* mutation demonstrated the AtPPME1 involvement in determination of the growth rate and the shape of the pollen tubes in *Arabidopsis*. Mutant *atppme1* plants displayed slower growth and irregular shape of pollen tubes grown *in vitro*, but retained the wild-type morphology of ungerminated pollen grains. Similarly to the *vgd1* mutant plants, the homozygous *atppme1* mutant pollen exhibited a 20% decrease in the overall PME activity, but, unlike the *vgd1* mutant, the *atppme1* plants displayed no apparent effects on stability of *in vitro*-grown pollen tubes or on plant fertility (Tian et al., 2006).

Because both *vgd1* and *atppme1* mutations resulted in comparable reductions in the overall pollen PME activity, but only *vgd1* caused pollen tube instability and led to male gametophytic defects (Jiang et al., 2005; Tian et al., 2006), VGD1 and AtPPME1 (and, potentially, other pollen-specific PMEs) may play functionally different and non-redundant roles in pollen tube development and growth. Alternatively, the observed differences in pollen tube stability may arise from different *in vitro* pollen tube growth conditions, and VGD1 and AtPPME1 may in fact perform similar, but additive functions. In this scenario, one could speculate that a double *vgd1/atppme1* mutant would exhibit a cumulative phenotype of its parental mutations, i.e., pollen tubes that are even more slow growing, irregularly shaped, and unstable.

Another important insight into the pollen PME activity came from the study in which a heterologous PME from orange was exogenously applied to germinated pollen grains of *Lilium formosanum* and tobacco, resulting in thickening of the pollen tube cell wall at the tip, with the consequent inhibition of the pollen tube growth (Bosch et al., 2005). These observations suggest that the enhanced extracellular PME activity generates extra carboxyl groups on the pectin residues in the cell wall,

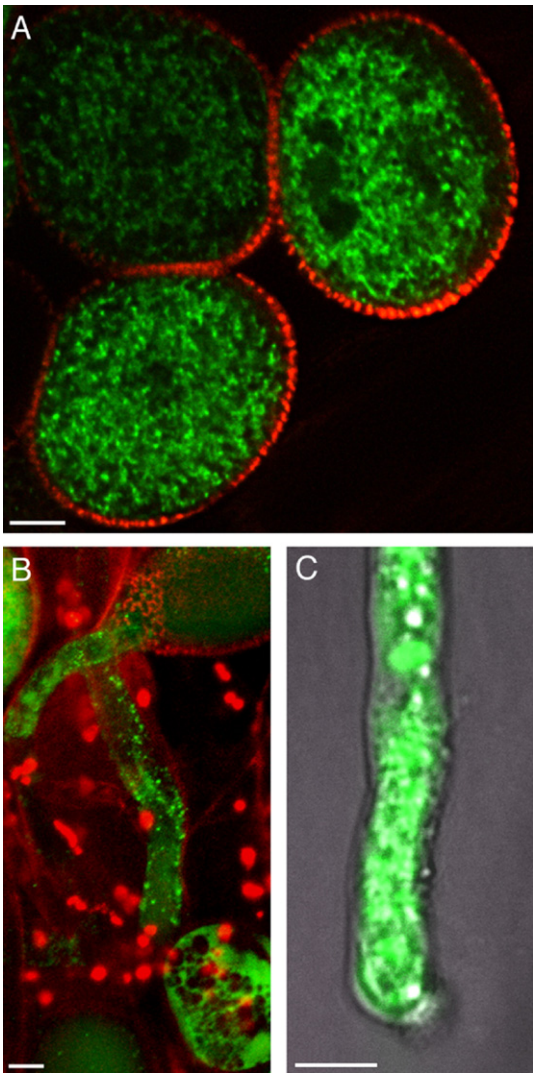

Fig. 1. Expression of *Arabidopsis* pollen-specific PME, AtPPME1, in pollen grains and pollen tubes. The full-length AtPPME1 was tagged with the yellow spectral variant of the green fluorescent protein (YFP) and transgenically expressed in *Arabidopsis* from its native regulatory elements; plant cell walls were visualized by staining with propidium iodide (Tian et al., 2006). (A) Mature pollen grains in an anther sac. (B) Pollen grains and tubes growing between stigmatic papilla in a living pistil. (C) Pollen tube germinated and grown for 24 h *in vitro*. YFP signal is in green, and plastid autofluorescence and propidium iodide signal are in red. Image in panel A is a single confocal section; images in panels B and C are projections of several confocal sections. Scale bars = 10 μm.

which are then cross-linked by calcium and thus contribute to the cell wall thickening and tube growth inhibition. Interestingly, PME applied "endogenously", i.e., by over-expression, caused a similar effect of inhibition of the tube growth, probably through similar changes in the mechanical properties of the apical tube wall. The same study also identified a novel tobacco pollen-specific PME, NtPPME1, and characterized this enzyme in respect to the activities of its pre-region and pro-region/PMEI domains (Bosch et al., 2005). Subsequent experiments demonstrated that NtPPME1 plays an important role in pollen tube growth as its silencing in tobacco retards the tube growth *in vivo*, albeit without detectible changes in the overall PME activity (Bosch et al., 2005). Thus, all studies of pollen-expressed PMEs point to a critical role of this class of cell wall-modifying enzymes in regulation of the pollen tube growth and morphogenesis.

Cellulose synthases

In addition to PMEs, cellulose synthases have been implicated in regulation of the pollen tube growth. The first plant cellulose synthase genes were identified through their high homology to cellulose synthases from bacteria (Pear et al., 1996). Cellulose synthases belong to a large family of β-glycosyltransferases, which includes mammalian hyaluronan synthases and fungal chitin synthases (Henrissat and Davies, 2000). There are about a dozen of cellulose synthase genes in *Arabidopsis* (Richmond and Somerville, 2000; Saxena and Brown, 2000), and computational analysis of the *Arabidopsis* genome indicated that a large number of additional uncharacterized sequences, termed *CELLULOSE SYNTHASE-LIKE* (*CSL*) genes, encode for putative glycosyltransferases (Hazen et al., 2002; Richmond and Somerville, 2000; Saxena and Brown, 2000).

At least one member of the *Arabidopsis CSL* gene family, a ubiquitously expressed *AtCSLA7*, has been shown to participate in the pollen tube growth, suggesting a requirement for β-linked polysaccharides for this process (Goubet et al., 2003). Reverse genetics experiments showed that male transmission efficiency of the mutagenic insertion in the *AtCSLA7* gene was significantly reduced in comparison to the wild-type plants, while female transmission was unaffected; this male gametophytic defect in the *AtCSLA7* gene mutant indicated the importance of AtCSLA7 for pollen functionality. Because in these mutant plants pollen tube guidance and fertilization were not affected, the mutation in *AtCSLA7* likely caused pollen tubes to grow slower and/or shorter in the pistil, precluding them from reaching the more distal ovules. The identity of the polysaccharide synthesized by AtCSLA7 and the detailed mechanism by which AtCSLA7, a ubiquitously expressed protein, affects the pollen tube growth, however, remain enigmatic (Goubet et al., 2003).

The function of the cell wall-modifying enzymes in pollen tube growth is inherently dependent on transport of the cell wall components (including the enzymes themselves) to the tip of the pollen tube (reviewed in Hepler et al., 2001; Steer and Steer, 1989) whereas the polarity of the tip growth requires actin (reviewed in Vidali and Hepler, 2001). Both vesicular transport and the actin microfilament dynamics are controlled by small GTPases belonging to the Ras superfamily (Cole and Fowler, 2006; Gu et al., 2004; Molendijk et al., 2004; Zheng and Yang, 2000a,b), making these small regulatory proteins key players in the pollen tube growth events.

Rab and Rop/Rac small GTPases: regulation of membrane vesicle traffic and actin dynamics

Small GTPases of the Ras superfamily are low molecular mass signaling proteins with regulatory activity, which depends

on their association with GTP or GDP. Historically, small GTPases have been viewed as binary molecular switches, with 'on' and 'off' positions being their GTP- and GDP-bound states, respectively. In their active, GTP-bound form, these proteins activate downstream effectors until they are converted into an inactive, GDP-bound form via their own GTPase activity. The intrinsic guanine nucleotide exchange and hydrolysis activities of some small GTPases, such as Ran, are weak, and their interactions with downstream effectors are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Meier, 2005). Small GTPases regulate a wide range of cellular eukaryotic processes, from membrane fusions (Gournier et al., 1998) to nuclear import (Steggerda and Paschal, 2002). In pollen tubes, they have been implicated in at least two distinct and vital functions: regulation of membrane vesicle trafficking and regulation of actin microfilaments.

GTPases: regulation of membrane trafficking

Rapid and polarized pollen tube growth demands an active secretory system which can provide a large amount of material needed to support the forward progression of the tube. Hence, exocytosis is one of the major processes driving the polarized cell growth (reviewed in Hepler et al., 2001; Steer and Steer, 1989), providing the growing tip with cell wall components, membrane material, enzymes, and receptor molecules (Muschietti et al., 1998) that sense female signals (Cheung et al., 1995), and more. Interestingly, despite the obvious prevalence of the exocytotic processes at the tip elongation site, endocytosis was also found to occur at the tip of the growing pollen tube – as shown using internalization of fluorescently labeled impermeant dyes (Carroll et al., 1998; O′ Driscoll et al., 1993) – and it was proposed to be involved in the retrieval of the excess cellular materials and in membrane turnover (Derksen et al., 1995; Picton and Steer, 1983). By maintaining a fine balance between the rates of anterograde and retrograde vesicle trafficking, both growth and recycling of membrane components are achieved, making these two processes a major driving force within the expanding region of the cell membrane of the pollen tube.

Both exo- and endocytosis are governed by a membrane fusion machinery which is strikingly similar between plants other eukaryotes (reviewed in Battey et al., 1999; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999). For example, plants encode homologs of the mammalian SNARE complex that are involved in fusion between vesicles and the target membrane (The Arabidopsis Genome Initiative, 2000). Furthermore, although plants do not contain true Ras GTPases (Vernoud et al., 2003), they encode homologs of mammalian and yeast Rab and Ypt, respectively, members of a large family of Ras-related small GTPases that regulate anterograde and retrograde vesicle transport between endomembranes and the plasma membrane (Rutherford and Moore, 2002; The Arabidopsis Genome Initiative, 2000; Vernoud et al., 2003). Although rapid growth and a highly dynamic intracellular trafficking system targeting the transport vesicles to the tip area are the hallmark characteristics of growing pollen tube, little had been known about how these processes are regulated in the pollen cell. In recent years, however, a substantial progress has been made in our understanding of the membrane fusion processes involving small Rab GTPases in the pollen (Cole and Fowler, 2006; Molendijk et al., 2004).

Like other members of the Ras GTPase superfamily, Rab proteins cycle between two distinct conformations, a GDP-bound, mainly inactive cytosolic form, and a GTP-bound, membrane-associated active form. Protein complexes formed in response to Rab activation perform diverse functions in membrane metabolism, including coupling of endomembranes to the cytoskeleton, as well as long-range vesicle docking/tethering interactions that regulate membrane fusion activity mediated by the SNARE machinery (Olkkonene and Stenmark, 1997; Pfeffer and Aivazian, 2004). Similarly to other small GTPases, Rabs have weak intrinsic guanine nucleotide exchange and GTP hydrolysis activities, and therefore their interactions with other proteins are regulated by GEFs and GAPs (Meier, 2005).

Being by far the most complex among all families of Ras proteins (Pereira-Leal and Seabra, 2001; Stenmark and Olkkonen, 2001), numerous Rab homologs have been identified in various plant species, including 57 Rabs in *Arabidopsis* (Rutherford and Moore, 2002; Vernoud et al., 2003). Associating with different endomembrane compartments, they regulate membrane budding, manage vesicle transport to the target areas and promote vesicle and target membrane fusion, thus being vital components for the transport of vesicles through the endomembrane system (Armstrong, 2000; Sanderfoot and Raikhel, 1999; Schimmoller et al., 1998; Seabra and Wasmeier, 2004; Tamm et al., 2003; Zerial and McBride, 2001). Several findings suggest that members of the Rab GTPase family play a crucial role in regulation of pollen tip growth. A cDNA of Rab GTPase, belonging to the Rab11 subfamily and designated NtRab11b, was isolated from tobacco pollen (Haizel et al., 1995) and shown to reside mainly in the transport vesicle-occupied apical clear zone of the growing pollen tip, providing evidence for a vesicular association of this protein (Derksen et al., 1995; Lancelle and Hepler, 1992). This localization of NtRab11b was dispersed by treatment with the actin-depolymerizing drugs, suggesting that NtRab11b might be associated with transport vesicles that track along actin filaments (Hepler et al., 2001). Furthermore, NtRab11b may be involved in endocytosis as functional inhibition of NtRab11b abolished the apical accumulation of extracellular fluorescent impermeant dye (Haizel et al., 1995).

Correct targeting and localization of NtRab11b likely depend on proper regulation of its GTPase activity by GEFs and GAPs because expression of a NtRab11b dominant-negative mutant, locked in its GDP-bound form, completely blocked the apical accumulation of NtRab11b while a constitutively activating mutation, locking the protein in its GTP-bound form, rendered NtRab11b more evenly distributed within the apical dome (de Graaf et al., 2005). Potentially, the dominant-negative NtRab11b mutant remains associated with the donor membranes, resulting in its primarily Golgi localization and inability to traffic to the tube apex. Conversely, the constitutive active NtRab11b mutant

most likely is released from the target membranes very slowly, resulting in a more uniform tip staining. Expression of either dominant-negative or constitutive active mutants of NtRab11b inhibited *in vitro* pollen tube elongation and directionality, and the expressing plants exhibited reduced male fertility. For the dominant-negative NtRab11b-expressing pollen, the defect likely stemmed from the combined effects of reduced tube growth rates and compromised tube capacity to penetrate ovules whereas, for the constitutive active NtRab11b-expressing pollen, the severely reduced fertility likely resulted from inhibition of pollen germination and tube growth (de Graaf et al., 2005). Thus, a correct ratio of active to inactive forms of NtRab11b must be stringently maintained for the optimal vesicular transport activity at the pollen tube tip. Collectively, the studies of NtRab11b established that properly regulated membrane trafficking activity is a key to rapid, tip-focused pollen tube growth and male fertility and, ultimately, successful plant sexual reproduction.

Another small Rab GTPase from tobacco pollen, NtRab2, was shown to function in the secretory pathway between the ER and the Golgi (Cheung et al., 2002). NtRab2 was strongly expressed in the pollen, as well as in other rapidly growing tissues with elevated demands for secretory activities, such as young seedlings, elongating hypocotyls, emerging true leaves and developing roots. On the other hand, the NtRab2 transcripts were not detectable in mature tissues, such as full-grown leaves (Cheung et al., 2002). In pollen tube, NtRab2 localized to the Golgi bodies, and its dominant-negative, inactive mutant blocked the delivery of Golgi-resident, as well as secreted marker proteins to their normal locations, consequently halting the pollen tube growth. When NtRab2 was over-expressed in transiently transformed leaf protoplasts and epidermal cells, in which the endogenous NtRab2 mRNA is not detected, it was not efficiently localized to the Golgi bodies, indicating that NtRab2 may be specialized to function mainly in the high secretory cell types, and that other cells may not possess components required for the normal function of this GTPase (Cheung et al., 2002).

GTPases: regulation of actin dynamics

In addition to Rabs, Ras-related GTPases belonging to the Rop (Rho of plants) – also referred to as Rac (Gu et al., 2004; Winge et al., 2000) – family are involved in the pollen tube growth (Chen et al., 2002, 2003; Gu et al., 2003, 2004; Li et al., 1999; Winge et al., 2000; Zheng and Yang, 2000a). The *Arabidopsis* genome encodes 11 Rops/Racs (Gu et al., 2004; Winge et al., 2000; Zheng and Yang, 2000b) belonging to four phylogenetic groups – group I (AtRop8), group II (AtRop9–AtRop11), group III (AtRop7) and group IV (AtRop1–AtRop6) – and members of the same group often exhibit functional redundancy, while members of different groups may perform distinct functions (Gu et al., 2004). The role of Rops/Racs in pollen development is well studied and, for its detailed description, the reader is referred to recent reviews (Gu et al., 2004; Zheng and Yang, 2000a, b). Briefly, in *Arabidopsis*, AtRop1, AtRop3, AtRop5, AtRop9–AtRop11, and AtRop8 are expressed in pollen (Gu et al., 2003, 2004; Li et al., 1998), and several of them have been implicated in regulation of tip actin dynamics and, possibly, regulation of calcium ion gradients. For instance, AtRop1 has been shown to regulate pollen germination and tube growth (Fu et al., 2001; Kost et al., 1999; Li et al., 1999). Because Rho GTPases regulate actin organization in yeast and animals (e.g., Kuhn et al., 2000), a possible role for AtRop1 in the similar processes in the pollen was examined (Fu et al., 2001). The findings suggested that AtRop1 activation was required for the tip actin assembly, and that the Rop1 signaling at the pollen tube tip regulated actin dynamics (Fu et al., 2001). The role of actin microfilaments in the pollen tube physiology is underscored by the recent observations that pharmacological disruption of actin polymerization inhibits pollen tube growth (Fu et al., 2001; Vidali and Hepler, 2001; Vidali et al., 2001), while promoting actin polymerization and stabilization of actin microfilaments by jasplakinolide promotes reversed cytoplasmic streaming in the apex of pollen tubes (Cárdenas et al., 2005). The proposed model for the AtRop1 action, therefore, suggests that the localization of the active Rop1 signaling complex defines and establishes tip growth domain and regulates the actin microfilament dynamics in the growing pollen tube (Gu et al., 2003). However, the exact mechanism of the AtRop1-mediated regulation of actin assembly, as well as the potential involvement of Rops in formation and regulation of the calcium ion gradient within pollen tube remains elusive (Gu et al., 2003; Li et al., 1999).

Several other members of the Rop/Rac family, such as the *Arabidopsis* AtRop9/AtRac7 GTPase, also affect pollen tube growth. Over-expression of AtRac7 in *Arabidopsis* induced a wide range of severe pollen phenotypes, which affected mostly the extent of the tube growth and intracellular organization and reduced the seed set in transgenic plants, further illustrating the importance of the Rop/Rac GTPase activity for pollen development and plant sexual reproduction (Cheung et al., 2003). Also, over-expression of the *Arabidopsis* AtRop1/AtRac11 (Fu et al., 2001; Li et al., 1999) and AtRac2 (Kost et al., 1999), as well as the tobacco NtRac1 (Chen et al., 2003), which is most similar to AtRop1/AtRac11 (Chen et al., 2003; Cheung et al., 2003), induced depolarized pollen tube growth most likely via actin depolymerization (Cheung et al., 2003) by an as yet unknown mechanism.

Taken together, these studies suggest involvement of two major families of small GTPases, Rabs and Rops/Racs, in the pollen tube growth. Although both protein families are related to each other, belonging to a single superfamily of Ras-related GTPases, their roles in the tube elongation process appear strikingly different. Specifically, pollen Rabs may function in vesicular transport and secretory processes in the nascent pollen tube. In contrast, pollen Rops/Racs likely participate in signal transduction and reorganization of actin microfilaments. Correct functionality of both Rabs and Rops/Racs is vital for proper pollen growth, because disruption of activity and/or expression levels of individual Rabs and/or Rops/Racs is detrimental to normal pollen development.

Whereas Rab and Rop/Rac GTPases regulate vesicular transport and actin microfilament dynamics, the actual movement

of the transport vesicles and, hence, pollen tube elongation, likely requires the action of diverse molecular motors (reviewed in Cai et al., 2000a). Unlike the actomyosin motility system, involvement of which in pollen tube growth is well described (e.g., Heslop-Harrison and Heslop-Harrison, 1989; Heslop-Harrison et al., 1988; Kohno et al., 1990; Tang et al., 1989a,b), the role of microtubule-associated molecular motors, i.e., dyneins and kinesins, has received less attention. Yet, increasing evidence indicates that both dynein and kinesin motors may fulfill an important function in vesicular transport within pollen tubes and in pollen tube growth (reviewed in Cai et al., 2000a; Moscatelli et al., 2003; Romagnoli et al., 2003b).

Microtubule-associated molecular motors: dyneins and kinesins

Vesicular transport in eukaryotic cells often relies on the dynamic interactions between motor proteins, anchored to the vesicle surface, and the cytoskeleton (reviewed in Allan and Schroer, 1999). Most likely, the movement of membrane vesicles and organelles during pollen tube growth is based on the same principles and thus involves microtubule-associated molecular motors (reviewed in Cai et al., 2000a; Moscatelli et al., 2003; Romagnoli et al., 2003b). These motor proteins – which biochemically represent enzymes that convert the chemical energy of ATP hydrolysis into mechanical force – belong to two major families: dyneins (Hirokawa, 1998; Holzbaur and Vallee, 1994; King, 2000, 2002; Moscatelli et al., 2003) and kinesins (Hoyt, 1994; Romagnoli et al., 2003b). Their main function is to translocate various cargoes, including vesicles and organelles, within a cell; in this intracellular transport system, dyneins and kinesins are thought to mediate movement in the opposite directions, i.e., toward the minus or the plus end of the microtubule, respectively (reviewed in Hirokawa, 1998; Moscatelli et al., 2003).

Dyneins

Dyneins, the molecular motor complexes of 1–2 MDa, are involved in various eukaryotic cell functions, including vesicle trafficking, mitotic spindle organization, Golgi maintenance and more (reviewed in Cai et al., 2000a; King, 2002). The motor activity of dyneins is promoted by their ~500-kDa heavy chains, which are responsible for interactions with microtubules and for ATP binding (Holzbaur and Vallee, 1994; King, 2000). The other components of the dynein complex, the intermediate and light chains, with molecular masses varying from 8 kDa to 80 kDa, are responsible for the motor–cargo interactions (Susalka et al., 2000).

Because vesicle secretion is one of the major mechanisms for the pollen tube elongation, it is not surprising that dynein heavy chain-like proteins were discovered in the pollen tubes of tobacco plants. These proteins interacted with microtubules in an ATP-dependent fashion (Moscatelli et al., 1995), and they contained epitopes immunologically similar to other dynein heavy chains (Moscatelli et al., 1998). Moreover, a sequence with 98% homology to the *Chlamydomonas* dynein heavy chain was amplified from a cDNA library of pollinated styles of tobacco. On the other hand, dynein light chains have not been identified in pollen; recent evidence, however, suggests that their functions may be carried out by the heavy chains themselves. This claim is based on immunological cross-reactivity between a dynein light chain from *Chlamydomonas* (LeDizet and Piperno, 1995a,b) and a 110-kDa polypeptide from tobacco pollen tubes (Moscatelli et al., 1995).

Kinesins

Kinesin-like proteins, which have been identified in most eukaryotes (reviewed in Barton and Goldstein, 1996; Hirokawa, 1998), usually comprise three functional and structural domains: a microtubule-binding force-generating head, a coiled-coil stalk region involved in dimer formation, and a cargo-binding tail domain. Kinesin-like proteins are involved in intracellular organelle transport, as well as mitotic and meiotic spindle movement and stabilization. In pollen, the first protein with biochemically distinct kinesin characteristics was identified in tobacco, where it localizes to the tube apex and associates with small vesicular structures (Cai et al., 1993; Tiezzi et al., 1992). A 100-kDa kinesin-related protein was then identified in hazel (*Corylus avellana*) pollen, where it was also found mainly in association with vesicles (Liu et al., 1994), lending further support to the notion that pollen kinesins mediate vesicular transport.

In recent years, additional microtubule-associated molecular motors from pollen have been discovered. For example, a 90-kDa polypeptide (ATP-MAP) from tobacco pollen released from microtubules by ATP exhibits kinesin-like properties, including association with microtubules in an ATP-dependent manner, enhanced microtubule binding in the presence of non-hydrolyzable ATP analogs, cross-reaction with antibodies against conserved kinesin domains and induction of microtubule gliding in an *in vitro* motility assay (Cai et al., 2000b). ATP-MAP was shown to bind to organelles associated with microtubules in the cortical region of the pollen tube, suggesting that this kinesin-related protein moves organelles in the tip region (Cai et al., 2000b).

Additional evidence of the involvement of kinesin-like molecular motors in pollen tube growth originates from the observations of movement of tobacco pollen organelles along *in vitro* polymerized microtubules in the presence of ATP. This movement was not dependent on addition of cytosol, suggesting that the motor proteins remained attached to the surface of the purified organelles. Indeed, following a potassium iodide wash to remove peripheral membrane proteins, the isolated organelles released factors that promoted gliding of carboxylated beads along microtubules and cross-reacted with anti-kinesin antibodies, leading to identification of a novel 105-kDa pollen-specific kinesin-related motor protein (Romagnoli et al., 2003a,b).

Interestingly, an important insight into pollen kinesins was gained through studies of "leaf hairs", the trichomes. Mutational analysis of the trichome development pathway identified a specific mutation, termed *zwichel* (*zwi*), which, besides producing trichomes with reduced branching and a shorter

stalk, affected the pollen tube growth (Folkers et al., 1997; Oppenheimer et al., 1997). The *ZWI* gene (Oppenheimer et al., 1997) encoded a kinesin-like calmodulin-binding protein (KCBP), a member of the kinesin family regulated by calmodulin (Narasimhulu et al., 1997; Oppenheimer et al., 1997; Reddy et al., 1996; Song et al., 1997). Subsequent genetic screen for suppressors of the strong *zwi*-3 allele identified three mutants, termed suppressors of *zwi*-3 (*suz*), one of which, *suz1*, displayed a synthetic sterile phenotype in the presence of the *zwi*-3 mutation (Krishnakumar and Oppenheimer, 1999). Because the parental *zwi*-3 and *suz1* plants were completely fertile, the reduced fertility of the *suz1/zwi*-3 double mutant suggests that the protein products of the corresponding genes may play roles in pollen development that are at least partially redundant to other cellular factors which can substitute for either, but not for both, of the *SUZ1* and *ZWI* functions. Reciprocal outcrosses of the *suz1/zwi*-3 mutants to the wild-type *Arabidopsis* plants failed to produce seeds when the *suz1/zwi*-3 mutant was used as the male, but not as the female parent, indicating that the reduced fertility of the double mutant plants was due to male sterility. No defects in reproductive systems of the *suz1/zwi*-3 mutants were observed, except for alterations in pollen germination *in vitro* (Krishnakumar and Oppenheimer, 1999). Specifically, germination frequency of the double mutant pollen was strongly reduced as compared to the wild-type pollen, and, even if *suz1/zwi*-3 pollen did germinate, their tubes were much shorter, aberrantly shaped and had anatomical anomalies, such as numerous spherical bodies, presumably vesicles, not observed in the wild-type or *zwi*-3 pollen tubes. Thus, the ZWI protein most likely represents a kinesin-like function involved in vesicular movement during pollen tube development and growth (Krishnakumar and Oppenheimer, 1999).

The directional pollen tube growth is achieved through a concerted action of cell wall-modifying enzymes and vesicular transport pathways which include regulatory Rab and Rop/Rac GTPases and microfilament- and microtubule-associated motility systems. A significant role in determination of pollen tube polarity belongs to intracellular distribution of cytosolic free calcium and such second messengers as phosphoinositides and cAMP (reviewed in Malhó et al., 2000).

Second messengers

PIP_2, IP_3, and phosphatidic acid

Phosphatidylinositol 4,5-bisphosphate (PIP_2) and its derivatives play roles in multitude of cellular processes, including cytoskeleton dynamics, vesicle trafficking, calcium ion homeostasis and others (Cremona et al., 1999; Stevenson et al., 2000), many of which represent important events in process of the pollen tube tip growth. Depending on the type of the phospholipase that uses PIP_2 as a substrate, it can be metabolized to smaller compounds that function as second messengers in the cell. For instance, inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) can be derived from PIP_2 by phospholipase C, and DAG can be further converted by a DAG kinase to phosphatidic acid (Munnik, 2001); alternatively, phosphatidic acid can be derived directly from PIP_2 through the action of phospholipase D (Powner and Wakelam, 2002).

Although IP_3 is involved in a variety of cellular processes, one of its major functions as a second messenger is regulating the release of calcium ions from internal storage compartments (Martin, 1998), which appears also to be one of its roles in the growing pollen tube (Malhó, 1998). Both PIP_2 and IP_3 were shown to be involved in maintenance of the calcium ion gradient and vesicle secretion in the apex of pollen tubes, and thus in regulation of the tip growth (Monteiro et al., 2005). When intracellular levels of PIP_2 and IP_3 in growing pollen tubes were altered using photolysis of caged PIP_2 and IP_3 osmotically loaded into *Agapanthus umbellatus* pollen tubes, tip-focused calcium gradient was modulated, reducing the rate of the tip growth and reorienting its axis (Monteiro et al., 2005). Interestingly, the detailed physiological effects caused by the increase of the intracellular levels of PIP_2 and IP_3, were somewhat different. The increased PIP_2 concentration inhibited apical secretion and reduced, but did not arrest, tube growth. The increase in IP_3, although causing a calcium spike similar in its magnitude to that induced by the elevated PIP_2, stimulated apical secretion and arrested tube growth (Monteiro et al., 2005). Several scenarios may explain these different effects of PIP_2 and IP_3; for example, the calcium release mechanisms for PIP_2 and IP_3 may vary, causing different cellular responses or the catabolism of PIP_2 can produce, in addition to IP_3, phosphatidic acid (see above) which, in turn, may generate additional metabolic effects.

In fact, phosphatidic acid has been shown to elicit strong physiologic effects on pollen tube growth (Monteiro et al., 2005). The reduction in the effective concentration of phosphatidic acid, promoted by antagonists of its accumulation (such as butanol) and phospholipase inhibitors, reversibly arrested development of tip polarity. On the molecular level, the reduction in phosphatidic acid levels caused immediate dissipation of the calcium ion gradient and inhibited apical plasma membrane recycling. Phosphatidic acid is known to stimulate translocation of calcium ions across cell membranes (Ohsako and Deguchi, 1981), which may explain its effect on the calcium gradient within the pollen tube tip. Yet, one cannot rule out other possible explanations, as essentially any stimulus leading to growth arrest has been found to disrupt calcium homeostasis (Holdaway-Clarke and Hepler, 2003), either because of a direct effect on calcium ion fluxes or as a consequence of some other indirect effect.

Phospholipase C

The implication of PIP_2 and its derivatives IP_3 and phosphatidic acid in regulation of the growth and axis orientation of the pollen tube tips places phosphoinositides and lipids as key regulators of tip growth. Thus, phospholipases probably are also involved in this process, since *in vivo* IP_3 and, ultimately, phosphatidic acid arise from the hydrolysis of PIP_2 by these enzymes. Supporting this notion, a novel phospholipase C, Pet PLC1, was discovered in the pollen tubes of *Petunia inflata*. The protein was expressed

predominantly in the anther/pollen and localized mainly to the apical plasma membrane of the pollen tube (Dowd et al., 2006). A catalytically inactive mutant of Pet PLC1 was expressed in nascent pollen tubes of *Petunia*, producing a dominant-negative phenotype with delocalized apical calcium ion gradient, and eventually resulting in disrupted actin cytoskeleton, arrested tube growth and swelling of the pollen tube tip. Also, over-expression of the C2 calcium/phospholipid binding domain of Pet PLC1 essentially phenocopied the expression of the inactivated Pet PLC1, suggesting that the C2 domain may interfere with the regulation of the endogenous enzyme and, thus, with calcium and/or phospholipid binding. On the other hand, expression of the C2 domain altered membrane association of the full-length Pet PLC1, raising a possibility that C2 simply displaces the full-length enzyme from its native location in the membrane (Dowd et al., 2006). Intriguingly, the phenotypic effects of expression of C2 or the inactive Pet PLC1 mutant could be suppressed by actin depolymerization, but they were unaltered by changes in extracellular calcium levels. Thus, Pet PLC1 likely regulates growth of the pollen tube tip via modification of actin dynamics, rather than by directly altering calcium fluxes (Dowd et al., 2006). Because Pet PLC1 shares a significant degree of homology with phospholipases C from other plant species, e.g., *Arabidopsis* phospholipase C2 (AtPLC2) (Dowd et al., 2006), this type of enzymes may represent an important and general element of the cellular machinery for controlling apical extension of the pollen

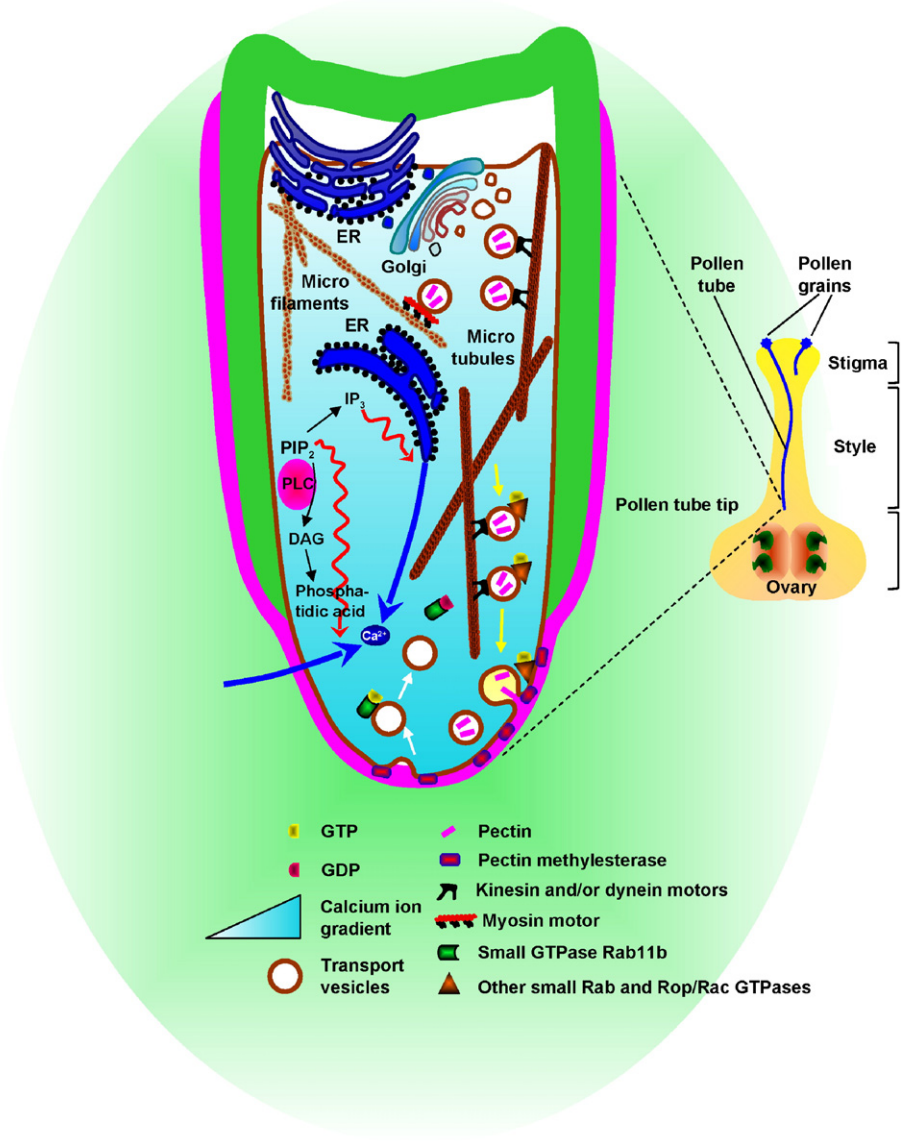

Fig. 2. Summary of the major roles of cell wall-modifying enzymes, small GTPases, molecular motors, vesicular transport, and second messengers in the growth of the pollen tube tip. Pollen grains land on the stigma, germinate, and their tubes grow through the spaces between the cells of the style until the pollen tip reaches the ovary and fertilizes the egg. During pollen tube growth, actin microfilaments, and probably microtubules, provide biological framework for heavy vesicular trafficking, as well as mechanical support, toward the elongating pollen tip. The corresponding molecular motors – myosin, dyneins, and kinesins – mobilize transport vesicles laden with cell wall materials. Rab and Rop/Rac GTPases regulate membrane fusion events and signaling processes. Second messenger-producing enzymes, e.g., phospholipase C, provide signaling molecules, such as DAG and IP_3 which, together with calcium ion gradient, regulate and direct pollen tube growth. A cell wall-modifying enzyme pectin methylesterase (PME) deesterifies pectins, which then are cross-linked by calcium ions, creating a new layer of pectin.

Table 1
Summary of cell wall modifying enzymes, small GTPases, molecular motors, and second messenger-producing/sensing proteins involved in pollen tube growth in various plant species

Protein class and cellular function	Protein name	Species	Effect in pollen tube growth	Reference
Pectin methylesterases (PMEs) Demethylesterification of homogalacturonan component of pectin during cell wall synthesis	Vanguard 1 (VGD1)	*Arabidopsis thaliana*	Pollen-specific PME that determines mechanical properties of the cell wall, morphology and growth of the pollen tube	(Jiang et al., 2005)
	AtPPME1	*A. thaliana*	Pollen-specific PME that determines growth rate and morphology of the pollen tube	(Tian et al., 2006)
	NtPPME1	*Nicotiana tabacum*	Pollen-specific PME required for pollen tube growth	(Bosch et al., 2005)
Cellulose synthases Assembly of cellulose microfibrils	AtCSLA7	*A. thaliana*	Ubiquitously expressed β-glycosyltransferase that affects late stages of growth and/or termination of extension of the pollen tube	(Goubet et al., 2003)
Rab GTPases Regulation of vesicular transport	NtRab11b	*N. tabacum*	Pollen-specific small GTPase involved in endocytosis at the pollen tip; over-expression inhibits pollen tube growth	(de Graaf et al., 2005; Haizel et al., 1995; Hepler et al., 2001)
	NtRab2	*N. tabacum*	Pollen-specific small GTPase that functions in secretary pathway between ER and Golgi and is required for pollen tube growth	(Cheung et al., 2002)
Rop/Rac GTPases Regulation of various cellular processes, including signal transduction and cytoskeleton organization	AtRop1	*A. thaliana*	Pollen-specific small GTPase that regulates pollen germination and tube growth	(Fu et al., 2001; Kost et al., 1999; Li et al., 1999)
	AtRop9/AtRac7	*A. thaliana*	Pollen-specific small GTPase, over-expression of which alters pollen tube growth	(Cheung et al., 2003)
	AtRop1/AtRac11, AtRac2	*A. thaliana*	Pollen-specific small GTPases, over-expression of which induces depolarized pollen tube growth	(Fu et al., 2001; Kost et al., 1999; Li et al., 1999)
	NtRac1	*N. tabacum*		(Chen et al., 2003; Cheung et al., 2003)
Microfilaments Cytoskeleton	Actins, myosins	All plants	Actin microfilaments and myosin motors are essential for vesicular transport and pollen tube growth	(Cai et al., 2000a; Geitmana et al., 2000; Hepler et al., 2001; Vidali and Hepler, 2001; Vidali et al., 2001)

Microtubules Cytoskeleton	Tubulin	*Picea abies*, *Nicotiana sylvestris*	Disruption of microtubule network partially blocks pollen germination and growth	(Anderhag et al., 2000; Joos et al., 1994)
Dyneins Microtubule-associated motor proteins	Dynein heavy chain-like protein	*N. tabacum*	Molecular motors are involved in vesicle/organelle movement and required for pollen tube growth	(Moscatelli et al., 1995, 1998)
Kinesins Microtubule-associated motor proteins	Kinesin-like proteins (ATP-MAP, 100-kDa and 105-kDa kinesin-related proteins)	*N. tabacum*, *Corylus avellana*		(Cai et al., 1993, 2000a; Liu et al., 1994; Romagnoli et al., 2003a,b; Tiezzi et al., 1992)
Kinesin-like calmodulin-binding proteins (KCBPs) Calmodulin-regulated microtubule-associated motor proteins	Zwichel (ZWI)	*A. thaliana*	(Folkers et al., 1997; Oppenheimer et al., 1997).	
(Sequence unknown) Genetically interacts with ZWI	Suppressor of ZWI (SUZ1)	*A. thaliana*	A suppressor of mutations in *ZWI*	(Krishnakumar and Oppenheimer, 1999)
Phospholipase C Metabolizing of fatty acids and production of second messengers DAG and IP_3	Pet PLC1	*Petunia inflata*	Regulation of pollen tube growth by modification of actin dynamics	(Dowd et al., 2006)
	AtPLC2	*A. thaliana*	Pet PLC1 homolog potentially involved in pollen tube growth	(Dowd et al., 2006)
Adenylyl cyclase Produces second messenger cAMP	Pollen signaling protein (PSiP)	*Zea mays*	Regulation of pollen tube morphology and growth rate	(Moutinho et al., 2001)
Calmodulin Calcium-binding protein that acts as primary sensor of calcium	Calmodulin	*Agapanthus umbellatus*	Regulation of pollen tube growth and polarity, likely via secretary pathways; activity modulated by cAMP	(Rato et al., 2004)

tube, further emphasizing the role of the calcium/phospholipid signaling system in the pollen tube growth.

cAMP

IP_3 also plays a key role in the regulation of another prototypical second messenger, cAMP, produced by adenylyl cyclases and degraded by phosphodiesterases (Bruce et al., 2002). Although several cAMP-dependent processes have been described in plants (Assmann, 1995; Bolwell, 1995), our understanding of the full extent of the biological effects of cAMP signaling on plant physiology is still incomplete. Imaging of cAMP distribution in living pollen tubes in *A. umbellatus* revealed that altering the resting concentration of cAMP alters the directional growth of the pollen tube tip. Specifically, when placed extracellularly, in the vicinity of an *in vitro*-growing pollen tube, chemicals that elevate the intracellular cAMP, e.g., dibutyryl cAMP and forskolin, attracted the growing tip whereas cAMP antagonists, e.g., theophylline and Rp-8-Br-cAMPS, had the opposite effect on the direction of the pollen tip growth (Moutinho et al., 2001).

Interestingly, although modification of cAMP levels within pollen tubes was accompanied by changes in calcium concentration (Malhó et al., 2000), cAMP was distributed throughout the pollen tube uniformly (Moutinho et al., 2001), and it did not form a tip-focused gradient described for cytosolic calcium ions (reviewed in Holdaway-Clarke and Hepler, 2003). Potentially, cAMP levels in the tip area are altered only transiently due to localized changes in the enzymatic activities of adenylyl cyclases and/or phosphodiesterases (Moutinho et al., 2001). Supporting this idea, a novel pollen signaling protein (PSiP) with homology to fungal adenylyl cyclase was identified in maize. PSiP produced cAMP when expressed in bacteria; furthermore, PSiP-specific antisense oligonucleotides altered the morphology and retarded the growth of pollen tube tips, and these effects were reversed by external application of cAMP and mimicked by cAMP antagonists (Moutinho et al., 2001).

The effects of cAMP on the pollen tube growth may, at least in part, occur via modulation of calmodulin activity (Rato et al., 2004). Calmodulin activity in the pollen tubes of *A. umbellatus* exhibited a tip-focused gradient (Rato et al., 2004), similar to that of cytosolic calcium ions (reviewed in Holdaway-Clarke and Hepler, 2003). Moreover, changes in the calmodulin activity within the tube apex reoriented its growth axis, thus suggesting that calmodulin may be involved in guiding polar growth of the pollen tube. Although till now the detailed mechanism by which calmodulin affects the tube growth process remains obscure, calmodulin activity may depended not only on calcium, but also on the intracellular levels of cAMP, indicating a cross-talk between different signaling pathways in controlling the pollen tube growth (Rato et al., 2004).

Concluding remarks

Directional growth of pollen tube through the pistil toward the ovary is critical for egg fertilization. Understanding the biosynthetic and regulatory pathways that govern the pollen tube growth, therefore, is central for understanding many aspects of plant sexual reproduction, from its molecular mechanisms to evolution. Recent progress in the studies of pollen tube growth discussed in this review uncovered a plethora of basic cellular processes, such as control of cell shape and growth by a network of cell wall-modifying enzymes, molecular motor-mediated vesicular transport along cytoskeletal tracks, and intracellular signaling by localized gradients of second messengers; the highlights of these processes are summarized in Fig. 2 whereas its protein participants are listed in Table 1.

As expected from good science, studies discussed in this review posed new challenging questions regarding the molecular mechanisms underlying the ability of pollen tubes to undergo guided, polar, and rapid elongation. Future studies will need to address, for example, a relatively unusual situation when multiple members of a protein family expressed in the same cell, i.e., pollen-expressed PMEs, fulfill apparently non-redundant functions, and how these enzymatic activities are regulated to allow directional growth of the pollen tube. Also, the fascinating ability of pollen cells to maintain tip-focused gradients of secondary messengers needs to be understood, and the interrelationships and cross-talk between these signaling systems unraveled.

The complexity of the cellular systems utilized by the pollen for its tube growth is just beginning to emerge; for example, recent studies suggest involvement of ubiquitination pathways (Huang et al., 2006) and calcium-regulated phosphorylation/dephosphorylation cycles (Yoon et al., 2006). Solving the intricate mechanisms of the pollen tube elongation will unravel principles that govern cell expansion and directionality of cell growth, significantly impacting and advancing many other fields of biological research, such as physiology of polarized neuronal cells.

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