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Abstract

The pollen tube is fundamental for the reproduction of seed plants. Characteristically, it grows relatively quickly and uni-directionally ("polarized growth") to extend the male gametophyte to reach the female gametophyte. The pollen tube forms a channel through which the sperm cells move so that they can reach their targets in the ovule. To grow quickly and directionally, the pollen tube requires an intense movement of organelles and vesicles that allows the cell's contents to be distributed to sustain the growth rate. While the various organelles distribute more or less uniformly within the pollen tube, Golgi-released secretory vesicles accumulate massively at the pollen tube apex, that is, the growing region. This intense movement of organelles and vesicles is dependent on the dynamics of the cytoskeleton, which reorganizes differently in response to external signals and coordinates membrane trafficking with the growth rate of pollen tubes.

Keywords: Actin filaments; cytoplasmic streaming; microtubules; motor proteins; tip growth

INTRODUCTION

The intracellular trafficking of vesicles and organelles is essential for cell growth and development. Although this process occurs in all eukaryotic cells, it is peculiarly relevant throughout the life cycle of plant cells and becomes more prominent as the plant cell enlarges (Verchot-Lubicz and Goldstein 2010; Tominaga et al. 2013). In typical mature plant cells, the large vacuole confines the cytoplasm to the cell cortex and to cytoplasmic strands running through the cell; this organization forces plant cells to actively move the organelles. Because efficient distribution of small and large molecules is crucial for cell viability, plant cells have developed cytological and molecular mechanisms to support intracellular motility. Consequently, this trafficking allows large plant cells to appropriately distribute molecules and organelles. However, intracellular trafficking is not a complete randomization (like a blender) and allows the non-uniform distribution of specific classes of organelles and vesicles. The unequal distribution of cell contents facilitates polar processes (such as polar auxin transport) that are the basis for morphogenesis. The requirement for both widespread distribution and specific accumulation can be easily observed in a specialized cell, the pollen tube. The pollen tube has a key role in the reproduction of seed plants as it carries the male gametes to the female gametophyte. The pollen tube of angiosperms and gymno- sperms has many features of a plant cell, such as an intense intracellular trafficking of organelles and vesicles. This process redistributes membrane-bound structures but it also allows the specific accumulation of secretory vesicles at the pollen tube apex, giving rise to a highly polarized cell. Although the pollen tubes of seed plants share the same function, the cytology of gymnosperm pollen tubes differs considerably from that of angiosperms (Justus et al. 2004; Chebli et al. 2013) such that a comparison between the two systems is difficult. Among angiosperm cell types, the pollen tube is unique. Although some other cell types, such as root hairs and certain cambial initials, have tip growth, the angiosperm pollen tube grows faster and longer than these other tip-growing cell types by orders of magnitude. Furthermore, the pollen tube expresses a host of proteins specifically. This review will therefore focus exclusively on the angiosperm pollen tube; comparison with other somatic cells will be done when appropriate. The core of the growth process in pollen tubes is supported by the selective transport and accumulation of secretory vesicles at the apical region. These vesicles contain mostly pectin (Gu and Nielsen 2013). In addition, the secretory process is balanced by endocytosis. Because a

secretory vesicle has a relatively high surface-to-volume ratio and because a large volume of cell wall material is needed, the apical region of the pollen tube sustains high rates of both endo- and exocytosis in essentially the same location. We know little about the signals that keep all of this vesicular traffic flowing smoothly.   The transport of organelles and vesicles depends on the cytoskeleton and is driven by motor proteins, mechano- enzymes that convert the energy of ATP hydrolysis into kinetic energy (Rogers and Gelfand 2000). In this way, the movement of organelles and vesicles occurs along specific tracks defined by the organization of the cytoskeletal elements. Therefore, to understand how the transport of membrane-bound structures takes place, it is necessary to analyze the structure and dynamics of the cytoskeleton.   This review will first describe the organization of the pollen tube cytoskeleton, how it promotes the trafficking of organelles and the local accumulation of specific organelle subsets in relation to pollen tube growth. We will cover microtubules briefly because available data are few. Next, we will treat the actin filament arrays and the actin motor protein, myosin. Subsequently, we will discuss how the cytoskeleton might restrict and control organelle movement in the pollen tube; more specifically, we will present information on how the cross-talk between actin filaments and microtubules might orchestrate a coordinated trafficking of organelles and vesicles to sustain the high growth rate of the pollen tube. In doing so, we will focus on specific organelle classes that show distinct pathways of movement. Finally, we will discuss how secretory vesicles move in the pollen tube tip, how this movement is supported by the cytoskeleton and how it is coordinated with growth rate.

THE CYTOSKELETON IN THE SHANKS OF POLLEN TUBES: MICROTUBULES AND THE ACTIN-MYOSIN INTERPLAY

The pollen tube can be schematically divided in at least two main districts, the non-growing region (called the shank) and the growing region, which is the domed apex (Figure 1). Although the whole apex grows, the rate is highest at the pole and falls to zero at the point of contact with shank. Cytologically, the apex is zoned, with the extreme apex containing vesicles and few if any organelles or cytoskeletal filaments (Hepler and Winship 2015). For simplicity, we will refer to this vesicle rich area as the "clear zone", although in some pollen tubes the clear zone extends well into the shank. In the basal region of the apex and extending a short way into the shank is a region that contains organelles and an actin rich structure, called the fringe. Even though it can extend into the shank, we will call this region the "sub-apex". Some models have the sub-apex as the peak region for exocytosis, but this is controversial and will be discussed further below. We will discuss the differential organization of the cytoskeleton in relation to distinct pollen tube domains while highlighting their possible interplay.   The cytoskeleton of pollen tubes comprises actin filaments and microtubules. The function of both cytoskeletal systems is largely comparable to that of other plant cells with a few exceptions. The cytoskeleton is organized to facilitate growth. As the pollen tube is a tip-growing cell, the cytoskeleton has been adapted for that purpose; its main function is therefore to deliver vesicles at the apex and to strengthen the cell wall behind the apex, maintaining the cylindrical shape of the cell. Unlike in animal cells, the movement of organelles in plant cells is predominantly based on actin filaments, while microtubules are more involved in cell wall assembly. Actually, these two functions are not separate and there exists a certain degree of overlap. In the pollen tube, the role of actin filaments is clearly related to the transport of organelles and vesicles while the role of microtubules is not well defined (Cheung et al. 2008). In the shank of the pollen tube, microtubules are mainly organized along the longitudinal axis, sometimes with a helical organization (Del Casino et al. 1993; Raudaskoski et al. 2001). Immunofluorescence images showed that microtubules are arranged in bundles throughout the pollen tube while electron microscopy images revealed the presence of lateral arms that cross-link microtubules (Lancelle et al. 1987). In the sub-apical and apical regions of pollen tubes, the organization of microtubules is less certain. In those regions, it is likely that microtubules are dynamic and therefore standard techniques of chemical fixation are likely to misrepresent the structure. Improved techniques of fixation have revealed a kind of tubulin fringe in the sub-apex of Lilium longiflorum and Nicotiana tabacum pollen tubes (Lovy-Wheeler et al. 2005); the function of this structure and its relationship with the actin fringe (see below) are not known. Due to this uncertainty, the presence and organization of microtubules in the apex of pollen tubes has always

been a matter of debate. In addition, the absence of recognizable effects caused by microtubule inhibitors on pollen tube growth reinforces the hypothesis that microtubules play subsidiary roles in growth (Heslop-Harrison et al. 1988). In contrast to actin filaments, there is little information on the presence and activity of microtubule-associated proteins (MAPs) in the pollen tube. The presence of MAPs is mainly inferred from data collected in other cells. Plant MAPs are a heterogeneous group of proteins involved in many facets of the microtubule's life, from polymerization and growth to alignment and severing, from the assembly of bundles to the dynamic interaction with actin filaments (Sedbrook and Kaloriti 2008). Because of the lack of data on pollen tube MAPs, little information is available on the dynamic control of microtubules. It is likely that microtubules are assembled in the sub-apex of Lilium auratum pollen tubes (Heslop-Harrison and Heslop- Harrison 1988) but more research is required to clarify this point. Interestingly, more information is available for microtu- bule motor proteins (kinesin). Because this protein family is also potentially involved in organelle movement, it will be discussed later in relation to the microtubule-actin interplay. The distribution of actin filaments has been the subject of many studies. By means of various labeling and visualization techniques, actin filaments were first observed in the form of bundles along the longitudinal axis of pollen tubes. It is now clear that the pollen tube is characterized by at least three different arrangements of actin filaments, which roughly correspond to the tip, the sub-apex, and the shank (Figure 1). Here, we describe the bundles of actin filaments in the shanks, as they are responsible for the long-range transport of organelles. In the next sections, the actin fringe of the sub- apex and the undefined actin array in the tip will be presented. The organization of actin filaments in the shank of the pollen tubes is relatively simple to describe and to understand in terms of function. The evenly dispersed array of fine actin bundles that run throughout pollen tubes are likely similar to the actin filament cables present in somatic plant cells, whose task is to promote the trafficking of organelles and vesicles (cytoplasmic streaming) (Shimmen 2007). Because organelle trafficking occurs in two directions (towards the apex and towards the grain), two hypotheses can reasonably be made. In the first, actin filaments are arranged with a single polarity (e.g., to facilitate the movement towards the apex) and the return movement is purely passive. In the second, actin filaments are present with opposite polarity capable of supporting the movement of organelles in both directions. Because speed and linearity of transport are similar in both directions, the latter hypothesis is more reasonable and thus actin filaments are somehow arranged with two distinct polarities, as shown in pollen tubes of Haemanthus albiflos (Lenartowska and Michalska 2008). The sub-apex of the pollen tube is where actin bundles converge into the actin fringe and where organelles reverse their movement and therefore has a critical role because either the assembly of actin filaments or the elongation of existing ones most likely occurs in that region (Cheung and Wu 2004; Hepler and Winship 2015). The assembly and the stabilization of actin filament bundles is due to the bundling   activity of various actin-binding proteins, including LIM proteins, villin, and SB41 (Vidali and Hepler 2001; Ren and Xiang 2007; Staiger et al. 2010; Huang et al. 2015).

THE SUB-APICAL ACTIN FRINGE AND ITS RELATIONSHIP TO ORGANELLE MOVEMENT

Actin filament bundles converge into a structure whose visualization (and therefore description) is not always straightforward: the so-called "actin fringe", a sort of ring or band of actin filaments that encircle the cell at the base of the dome (Figure 1). The visualization of the actin fringe has been optimized with the development of improved methods of fixation (Lovy-Wheeler et al. 2005) or by using transformed cells expressing actin-binding proteins fused with fluorescent markers (Vidali et al. 2009). The visualization of this structure with standard techniques of chemical fixation is quite complicated. Nevertheless, the actin fringe is considered a characteristic trait whose identification probably dates back to a work of Heslop-Harrison and Heslop-Harrison (1991). In that manuscript, a collar of actin filaments was sometimes observed in pollen tubes of Narcissus pseudonarcissus and hypothesized to filter the membranous material reaching the pollen tube apex. Over the years, the role of the actin fringe has become progressively clearer, even though its precise structure and composition has not yet been clearly defined. The actin fringe is likely to be a dynamic structure whose extension is closely related to the rate of pollen tube growth (Dong et al. 2012). The assembly of the actin fringe is likely the result of a balanced process of assembly/disassembly regulated by many factors and proteins. On one hand,

at the leading edge of the fringe, the high concentration of calcium there activates proteins such as profilin, gelsolin, and villin that might promote actin disassembly (Huang et al. 2004, 2015). Additionally, the ability of LIM proteins to form actin filament bundles at the apex is likely to be hindered by high concentration of protons (Wang et al. 2008). On the other hand, proteins such as fimbrin may contribute to stabilizing the actin fringe (Su et al. 2012) while assembly of actin filament bundles at the trailing edge of the fringe would be favored by the presence of the alkaline band through activation of actin depolymerizing factors (ADFs) (Lovy-Wheeler et al. 2006). Discussion of the proteins that regulate the assembly of actin filaments is, however, beyond this manuscript and readers may refer to a review by Ren and Xiang (2007). The actin fringe can be considered a kind of turning point in the cell because most of the larger organelles stop and reverse their movement in proximity of the actin fringe, while secretory vesicles can usually cross the actin fringe thereby reaching the apex. The molecular basis of the discriminating activity of the actin fringe is still enigmatic. We can speculate that the actin fringe passively discriminates membrane-bound structures on the basis of their size. In this model, the different organization of actin filaments in the fringe might be sufficient to hinder organelle movement allowing vesicles to travel to the apex. As an alternative hypothesis, the organelle filtering is active and the actin fringe dynamically transports and focuses secretory vesicles, while hindering larger organelles from reaching the apex (this will be discussed further in the section on vesicle flow in the tip). Although it is very likely that the actin fringe regulates the growth rate of the pollen tube, we do not know exactly whether the actin fringe negatively restricts the movement (and thus the fusion rate) of secretory vesicles or if it positively boosts vesicle flow. When the growth direction of Camellia japonica and L. longiflorum pollen tubes is arbitrarily imposed, the density of the actin fringe changes, becoming greater on the slowly growing side and lesser on the rapidly growing side (Bou Daher and Geitmann 2011). This suggests that changing the growth direction either requires or induces a re-structured actin fringe; if this were true, the predominant function of the actin fringe would be to restrict or redirect the vesicle flow (Figure 2). As an alternative model, we might assume that a higher density of the actin fringe would increase the vesicular traffic on the less-growing side thereby shifting the point of vesicle fusion towards that side (Bou Daher and Geitmann 2011). This uncertainty suggests that more information is required on the relationship between actin fringe and vesicle transport.

ACTIN FILAMENTS IN THE TIP?

The region at the very apex is probably characterized by a set of short, highly dynamic actin filaments (Lovy-Wheeler et al. 2005; Vidali et al. 2009). Actually, we do not know whether this arrangement is due to the dynamic nature of actin filaments or to a technical inability to properly image them. The involvement of these highly dynamic actin filaments in the final step of vesicle trafficking is not known and we consequently need more information on the possible interactions between these filaments and vesicles during secretion and endocytosis. We cannot reject an alternative and plausible hypothesis, namely that such short actin filaments are simply an intermediate pool in the assembly of more organized actin filaments for the fringe and play no role at the very apex. The apical plasma membrane and the cytoplasmic region immediately beneath the tip contain myriad regulatory factors necessary for growth of the pollen tube (calcium, phospholipids, GTPases, reactive oxygen species, actin-binding proteins, etc.; see below for additional information). This pool of molecules is probably the "control center" in which decisions are imple- mented about the direction and rate of pollen tube growth (Zonia 2010); therefore, actin filaments in the tip are expected to be highly dynamic to adapt quickly to the new growth conditions. As minimal quantities of latrunculin stop tube growth without apparently affecting organelle motility in the shank (Vidali et al. 2001), a sensitive sub-population of actin filaments in the apex or sub-apex is evidently involved in growth. Considering the distribution of actin filaments at the pollen tube apex and sub-apex, potential candidates for this function are both the actin fringe and the short dynamic actin filaments.

MYOSIN ACTIVITY IS THE DRIVING FORCE OF ORGANELLE MOVEMENT

As discussed above, the pollen tube is characterized by different actin arrays that are locally adapted to the specific functions they play. To promote the movement of membrane- bound structures, actin filaments must interact dynamically with a family of motor proteins, namely myosin. The directional driving force allows the movement of organelles and vesicles along actin filament bundles. Accordingly, depolymerization of actin filaments by chemical inhibitors blocks the movement in a dose-dependent manner (Vidali et al. 2001). Second, inhibitors of the enzymatic activity of myosin block the transport of vesicles and organelles (although inhibitors of myosin typically have off-target effects) (McCurdy 1999; Cai et al. 2011). Third, myosins are localized in association with pollen tube organelles in L. longiflorum, Nicotiana alata, and N. tabacum albeit with heterologous antibodies (Tang et al. 1989; Miller et al. 1995; Tirlapur et al. 1995). Fourth, a protein with the enzymatic activity of myosin has been isolated from lily pollen tubes (Yokota and Shimmen 1994). Plant myosins are represented by two families, class VIII and XI, which are responsible for the fast and long distance transport of membrane-bound structures on actin tracks. The total number of myosin genes is about 14–17 depending on the species. Several years of research have shown that the concept of "one myosin – one organelle" is unlikely to be correct and that the functions of single myosins probably overlap each other (Buchnik et al. 2015). This is particularly evident for myosins interacting with the Golgi apparatus; the analysis with partial myosin sequences fused to fluorescent markers suggest that several myosin isoforms are responsible for Golgi movement (Avisar et al. 2009), although it is difficult to draw firm conclusions from the expression of protein fragments because of the potential for neomorphic effects. The overlap of myosin function is probably necessary to achieve the full motility of organelles. In addition, different myosins might be required for specific but correlated activities such as tubular shaping and movement of the endoplasmic reticulum (Griffing et al. 2014). Genetic analysis performed with deletion mutants, gene knockout and protein-binding assays revealed the existence of new vesicular compartments to which specific myosins appear to be associated (Peremyslov et al. 2013). This suggests that the role of plant myosins cannot be deciphered by simply analyzing the main organelle classes. We have little clear information about the presence of different myosin isoforms in pollen tubes. Although immuno- logical data suggest the presence of multiple isoforms (Miller et al. 1995), this information has been supported by neither biochemical nor genetic data. In addition, the distribution of myosin in the pollen tube of N. tabacum and Tradescantia virginiana does not suggest specific isoform association with specific organelles (Yokota et al. 1995). The regulation of myosin activity is likely to depend on intracellular calcium concentration and on the associated calmodulin light chain, the latter functioning like a molecular switch. Changing to calcium concentration is expected to release calmodulin from myosin thereby inducing a conforma- tional change that affects myosin motility and cytoplasmic streaming (Tominaga et al. 2012). In L. longiflorum pollen tubes, the regulation of myosin activity is likely based on comparable mechanisms because a 170–175 kDa myosin is regulated by calcium through binding with calmodulin (Yokota et al. 1995). As the apex of pollen tubes is characterized by high levels of calcium, the most plausible hypothesis is that the enzymatic activity of myosin is inhibited at the pollen tube apex, where active transport of organelles and vesicles might not be required (Figure 1). However, this hypothetical mechanism controlling the enzymatic activity of myosin does not explain the behavior of organelles such as mitochondria and endoplasmic reticulum (which are likely to move along actin filaments). As these organelles are differentially distributed in regions where a gradient of calcium is absent, we might exclude a direct effect of calcium on the differential motility of these compartments. Where present, the calcium gradient affects the structure of actin filaments in addition to the enzymatic activity of myosin, thereby generating an indirect effect on organelle motility (Steinhorst and Kudla 2013). As the actin fringe is the area where larger organelles reverse their motion, we can speculate that the activity of myosin is somehow controlled at this level. At the apical part of the shank, the actin filament structure changes from longitudinal bundles to more irregular and twisted structures until converging into the actin fringe. The irregular arrange- ment of actin filaments basal of the fringe might affect organelle movement by interfering with the processivity of myosin. Because there are no data on the activity of organelle- associated myosins in the sub-apex and in relation to the actin fringe, one hypothesis is as good as any other.

DO MICROTUBULES AND ACTIN FILAMENTS COOPERATE FOR ORGANELLE TRANSPORT IN POLLEN TUBES?

In the previous sections, we discussed the organization of actin filaments in relation to the pollen tube cytology. We also stated that the movement of organelles in pollen tubes depends on actin filaments. Nevertheless, pollen tube organelles are not uniformly distributed, as it would be expected from the activity of cytoplasmic streaming alone (Hepler and Winship 2015). In the following sections, we present evidence on the uneven distribu- tion of some organelle classes. The differential positioning of organelles necessarily requires a regulatory mechanism, which might be conceivably based on the different organization of actin filaments, on the regulation of myosin activity and, possibly, on the cooperation between actin filaments and microtubules. The so-called "functional cooperation" implies that motor proteins of different families are simultaneously bound to the organelle surface where they might work synergistically or antagonistically. The synergistic activity of different motor proteins implies that one motor is required for a first motion step, while another motor is required for a second motion step; the two steps may be characterized by different velocities or because they occur in different cell areas. The antagonistic activity of motor proteins requires that one motor protein counteracts the propulsive activity of another motor resulting in a kind of "tug-of-war". Functional cooperation between microtubule- and actin-based motor proteins was first suggested in animal cells (Lambert et al. 1999) and in fungal cells (Steinberg 2000). In plants, organelles are recognized to move rapidly and for long distances along actin filaments by the propulsive activity of myosins (Shimmen 2007). On the other hand, microtubules and kinesins might slow down locally the transport speed of organelles, which consequently would accumulate in specific cell districts. Now, the question arises whether the mechanism of functional cooperation explains the uneven distribution of organelles as observed in pollen tubes. To understand this mechanism, it is necessary to determine the relationship between actin filaments and microtubules in pollen tubes. The two systems have a similar but not identical organization. The degree of overlap and alignment is not known although several datasets suggest that the two systems interact. Optical and electron microscopy images show the close alignment between actin filaments and microtubules (Pierson et al. 1989; Lancelle and Hepler 1991, 1992), while biochemical data reveal the existence of proteins that bridge the two systems (Huang et al. 2000; Romagnoli et al. 2010; Wang et al. 2013). Furthermore, the selective depolymerization of one of the two systems significantly impacts the other (Poulter et al. 2008). Although the number of microtubule-actin cross-linking proteins identified in plants is low (and their functions not clear), cross-talk between microtubules and actin filaments is likely to be involved in a variety of cell functions and features (Petrasek and Schwarzerova 2009). In the pollen tube, we need more data before we can conclude that actin filaments can affect the polymerization of microtubules. It is also poorly known whether the structure of actin filaments or microtubules changes along the axis of pollen tubes. It is generally assumed that these filaments are homogeneously distributed in the pollen tube shank but microtubule deployment in the pollen tube has yet to be mapped in detail. Therefore, we can only hypothesize that organelle transport along the pollen tube is impacted by the polymerization state of individual cytoskeletal filaments and by their reciprocal interaction. Both parameters could in turn affect the functional state of motor proteins. It is interesting to note that the in vitro movement of organelles from the N. tabacum pollen tube is affected by the relationship between actin filaments and microtubules. Individual organelles or vesicles can move quickly along actin filaments but, as soon as they approach a microtubule, their velocity slows down considerably (Romagnoli et al. 2007). Consequently, the model of functional cooperation may explain the unequal distribution of organelles in pollen tubes but more data are required to validate this model. The movement of plant organelles along microtubules is hotly debated. It is likely that microtubules are involved in specific trafficking events, such as the vesicle-based delivery of cell wall components at the phragmoplast (Lee et al. 2001). In many cases, a microtubule-based motility of organelles might be simply hidden by the considerable movement of organelles along actin filaments. In other cases, a microtubule-based motility of plant organelles may result in a kind of pausing rather than in directed movement. Pausing of plant cell organelles along microtubules is gradually being recognized as occurring commonly. Although microtubule inhibitors have little or no effects on the directed (i.e., long distance) motility of plant organelles, we cannot exclude that microtubules condition short-range

movement, which is difficult to charac- terize. In shoot epidermal cells of Arabidopsis, various organelles, including the Golgi, endoplasmic reticulum, perox- isomes, and RNA processing bodies, pause frequently along microtubules. Interestingly, the frequency of pausing is apparently independent on the integrity of microtubules (Hamada et al. 2012). This suggests that microtubules are required but not essential for organelle pausing and raises the question on the function of microtubule-based pausing. Pausing might be required for supporting organelle inter- actions or for facilitating local events, as outlined in the review of Brandizzi and Wasteneys (2013). For example, in diffuse growing cells, microtubules are required for the local insertion of cellulose synthase complexes in the plasma membrane or even for their recycling (Crowell et al. 2009; Gutierrez et al. 2009). We will return to this topic in the following sections in discussing the movement of individual organelle classes and by providing evidence for microtubule-actin interplay.

MOVEMENT AND DISTRIBUTION OF SPECIFIC ORGANELLES ALONG THE POLLEN TUBE

The activity of myosin in combination with the organization of actin filaments determines the traffic of organelles and vesicles along the pollen tube. The analysis of individual organelles allowed researchers to reveal some features of organelle movement. Membrane-bound structures were found to move directionally along approximately straight pathways. By tracking their paths along the main axis of the tube (Figure 3), we can see that organelles move linearly along invisible tracks, which probably represent actin filaments. In tobacco pollen tubes, organelle trajectories, plotted by using specific software, are correlated with the distribution of actin filaments (de Win et al. 1998). Movement is not at a constant rate but undergoes pulses of high speed followed by (usually very short) periods of lower speed. Myosin XI is a processive motor (Tominaga et al. 2003) for which we would expect a constant motility. However, it is also expected that the density of organelles within the tube is high enough to create steric hindrance capable of altering the processivity of movement. To determine which classes of organelles move along actin filaments and the kind of movement they exhibit, it is necessary to specifically label different organelles and to track their movement. This analysis was performed for mitochon- dria, endoplasmic reticulum, and vacuoles in the elegant work of Lovy-Wheeler et al. (2007) in which the authors analyzed organelle movement in pollen tubes of Lillium formosanum and L. longiflorum following treatment with microtubule- and actin filament-inhibitors. Organelle movement was found to be dependent on actin filaments because it was perturbed by latrunculin B. As anti-microtubule chemicals (such as oryzalin) did not have distinct effects, microtubules were suggested to be scarcely involved. In un-treated pollen tubes, despite the vigorous cytoplasmic streaming, each of the organelles maintains a unique distribution within the cell. The consequence of these findings is that the vigorous movement of organelles typical of cytoplasmic streaming does more than distribute them ubiquitously but also deploys them in a polarized or asymmetric manner. Given that actin filaments are apparently distributed evenly throughout the pollen tube (at least until the actin fringe), there must exist a molecular mechanism that allows the content of organelles to be differentially positioned. The candidates for this function can reasonably be the regulation of myosin activity, the local fine- scale organization of actin, and the dynamics of microtubules. Below, we discuss the features of the major pollen tube organelles (vacuoles, mitochondria, Golgi bodies and plastids) and of secretory vesicles; in addition, we debate how their movements and distributions might be regulated. Here, it is important to reiterate that pollen tube organelles usually show distinct velocities, which might also differ from species to species. Currently, there is no direct correlation between organelle velocity and growth rate of pollen tubes. By using minimal doses of actin drugs, Vidali et al. (2001) stopped growth without affecting organelle movement, but to our knowledge, the reverse (i.e., tube growth in the absence of organelle movement) has never been demonstrated. Trafficking of organelles is likely required to progressively accumulate the cytoplasm towards the growing region and to focus selectively on organelles in distinct cell regions, such as mitochondria that accumulate predominantly in the sub-apical region of pollen tubes.

Endosomes and pre-vacuolar compartments
The vacuole is likely formed by thin longitudinal and cross- linked tubules undergoing an active movement far from the apical region. Visualization of vacuoles, labeled with green fluorescent protein

(GFP)-tagged tonoplast proteins, confirmed that they have a tubular shape and are absent from the tip and sub-apex of Arabidopsis pollen tubes, although a more extensive vacuolization was observed in advanced stages of tube growth (Hicks et al. 2004). Like other plant cells, vacuoles likely have multiple functions in the pollen tube. This membrane compartment conceivably contributes to maintaining turgor pressure, which is required for tip-growth as for diffuse growth (Kroeger et al. 2011). An additional function of vacuoles is the degradation of proteins. A validated model of the interplay between endocytotic membranes in pollen tubes is not available; however, it is assumed that Golgi bodies deliver components to the trans- Golgi-network, which in turn provides secretory vesicles to the apical domain. Vesicles that do not fuse with the apical plasma membrane may be recycled directly within the apical dome and may attempt to fuse again; alternatively, unfused vesicles may go back to the trans-Golgi-network. This compartment would act as an intermediate in delivering membranes and unnecessary components to a further transitional compartment, called the pre-vacuolar compart- ment or, sometimes, the multi-vesicular body. The latter would finally deliver unnecessary material to the vacuole for degradation (Wang et al. 2011a). The use of positively or negatively charged nanogold particles allowed researchers to dissect the endocytotic process in N. tabacum pollen tubes and highlighted distinct pathways (Moscatelli et al. 2007). A first path for endocytosis of plasma membrane occurs in sub-apical domains that are recycled through the Golgi apparatus, while a second path mainly involves the retrieving of plasma membrane at the pollen tube tip. The two processes may or may not require the presence of clathrin (Moscatelli et al. 2007). This extensive exchange of membrane does not occur randomly but a precise communication must exist. Therefore, because of their intimate connection with endocytosis, it is not surprising that vacuoles do not exactly follow the movement of other membrane compartments (Lovy-Wheeler et al. 2007). The traffic of endocytotic vesicles is typically studied by analyzing specific proteins, such as the vacuolar sorting receptors, integral membrane proteins that mediate the transport of (extracellular or lumenal) proteins into the pre- vacuolar compartment. Analysis by confocal immunofluorescence and immunogold electron microscopy showed that these sorting receptors reach the cell membrane in pollen tubes (Wang et al. 2011a). This implies that they mediate the endocytotic uptake of soluble proteins and supports the existence of a continuous membrane flow to and from the plasma membrane to the pre-vacuolar compartment (Wang et al. 2011a). By examining the growth of Arabidopsis pollen tubes that express fluorescent protein markers specific for transport vesicles and endosomes, disorganization of mem- brane trafficking by brefeldin A (an inhibitor of anterograde flow from endoplasmic reticulum to the Golgi) was found to impair the motility of ARA7- but not ARA6-containing endo- somes (Zhang et al. 2010a). At the same time, actin filament inhibitors induced different effects on distinct endosomal populations, indicating that a dynamic actin cytoskeleton is essential for specific steps of the endomembrane traffic (Moscatelli et al. 2012). Although the role of microtubules in secretion and endocytosis has yet to be characterized carefully in pollen tubes, recent results show that the integrity of the microtubule cytoskeleton is important for both events. By inhibiting the organization of microtubules with nocodazole, it was inferred that microtubules play a role in promoting the invagination of the plasma membrane at the pollen tube apex thereby affecting the subsequent migration of vesicles from the apex for either degradation or recycling (Idilli et al. 2013). Further evidence for a role of microtubules in the internalization of plasma membrane domains came from studies of self- incompatibility in apple pollen tubes. In this case, inhibitors of microtubules were capable of blocking the effects of S-RNase, suggesting that microtubules participate in its internalization (Meng et al. 2014). Taken together, these data indicate that microtubules are likely to be critical for the internalization of plasma membrane (at least during self- incompatibility) while actin filaments play a major role in the trafficking of endocytotic or endosomal membranes. This may consequently represent an example of functional cooperation between microtubules and actin filaments in the pollen tube (as discussed above).

Mitochondria

Mitochondria move actively within the pollen tube but they are more concentrated in the sub-apical region of pollen tubes, probably to provide energy to the active growing region (Colaço et al. 2012). The hypothetical association between the differential distribution of mitochondria and the local produc- tion of energy in the sub-apex was suggested by analysis of NAD(P)H in L. formosanum pollen tubes

(Cardenas et al. 2006). NAD(P)H is a coenzyme required for the synthesis of ATP and its distribution overlaps with that of mitochondria suggesting that these organelles produce higher levels of ATP in the sub- apex. In terms of energy requirement, it would be important to understand the relationship between energy production and energy consumption required for actin dynamics in the sub- apex. This value has not yet been estimated but it is very likely that pollen tubes consume appreciable energy in the form of ATP because the ATP/ADP exchange is necessary for the polymerization of new actin filaments (Rounds et al. 2011b). Mitochondria are not usually found at the very tip of pollen tubes, perhaps because the activity of the actin fringe excludes mitochondria. If so, then specific behavior of actin filaments would underlie movement and positioning of mitochondria. In root hairs of Arabidopsis, treatment with actin filament- perturbing agents has considerable effects on the movement of mitochondria, suggesting that a fully functional actin cytoskeleton is necessary to support the motility of these organelles (Zheng et al. 2009). As the organization of actin filaments changes markedly in the sub-apical region with formation of the actin fringe, it is reasonable that this modification would affect the trafficking of mitochondria thereby constraining them to gather near the sub-apical region. If this be true, then the density of mitochondria in the sub-apex should be related to the density of the actin fringe, which, as discussed elsewhere, oscillates in relation to the growth rate of pollen tubes. Assuming that the local density of mitochondria can be correlated with the structure of actin filaments, the driving force promoting their movement is maintained by myosin. The association of mitochondria with myosin has been confirmed in several studies. First, mitochondria isolated from N. tabacum pollen tubes move actively along actin filaments and polypeptides immunologically related to myosin have been identified in mitochondrial fractions by immunoblotting and immunoelectron microscopy (Romagnoli et al. 2007). In addition, the binding of myosin to mitochondria has been demonstrated in other cell types using biochemical (Wang and Pesacreta 2004) and genetic analysis (Reisen and Hanson 2007; Avisar et al. 2008, 2009). In particular, in Arabidopsis six different myosins have been argued to be responsible for the motility of mitochondria and Golgi bodies (Avisar et al. 2009). In L. longiflorum pollen tubes, only one type of myosin XI has been identified (Yokota and Shimmen 1994) and demonstrated in association with mitochondria (Romagnoli et al. 2007). The movement of mitochondria in plant cells is quite peculiar. Generally, mitochondria can be classified as immobile or mobile. The relationship between these two pools is not clear but, apparently, there is an exchange between the two pools based on the organelle's metabolic state (Logan and Leaver 2000). Mitochondria mobility also seems to be affected by the cell's age (Van Gestel et al. 2002). The regulation of mitochondrial mobility depends on largely unknown factors; however, in Arabidopsis, implicated in mitochondrial shape and mobility are the "friendly mitochon- dria" genes, although their precise role remains to be elucidated (Logan et al. 2003). In diffuse growing cells, microtubules appear to take part in constraining or abolishing the movement of mitochondria, especially in the cell cortex (Van Gestel et al. 2002). Microtubules could anchor mitochondria, thus counteracting the driving force exerted by myosin. A higher density of mitochondria in the cortex could satisfy the energy demand of several activities such as synthesis, transport, and communi- cation occurring at the plasma membrane. To date, specific proteins anchoring mitochondria to microtubules have not been identified but there is evidence for the association between mitochondria and kinesins. In roots of Arabidopsis, specific kinesins have been identified in association with mitochondria, suggesting that microtubule motors can take part in the localization of these organelles (Ni et al. 2005). Interestingly, these kinesins interact with proteins involved in energetics (Yang et al. 2011), suggesting that microtubules influence the energy capacity of mitochondria in coordination with their position. In the pollen tube, mitochondria move mainly along actin filaments but a role for microtubules cannot be excluded. Application of the microtubule inhibitor, oryzalin, to N. tabacum pollen tubes changes the movement pattern of mitochondria by increasing the average speed and reducing the frequency of slowly moving mitochondria, suggesting that microtubules delay the movement of these organelles (Romagnoli et al. 2007). In support of this, kinesin-like proteins have been identified in association with pollen tube mitochondria (Romagnoli et al. 2007). The speed of these kinesins is much lower than cytoplasmic streaming. Comparably, mitochondria purified from tobacco pollen tubes move along microtubules during in vitro motility assays but the speed of movement is one-tenth the speed of the same organelles along actin filaments. Collectively, these data suggest that kinesins do not promote the movement of mitochondria but they might force them to pause, perhaps

in relation to energy demand. We do not know if microtubules have a role in gathering mitochondria in the subapical region of pollen tubes. As discussed above, the arrangement of microtubules in the sub-apex is unclear and more data are needed to understand mitochondrial positioning in the pollen tube.

Plastids
Understanding the movement of plastids in pollen tubes suffers from a shortage of information. However, the use of proteins targeted to plastids and fused with yellow fluorescent protein (YFP) recently allowed researchers to highlight the movement of plastids within the pollen tube of Arabidopsis thaliana (Fujiwara et al. 2012). The authors described at least three different types of movement: relative immobility, movement rapidly forward and backward, and stochastic movement, which occurred mainly at or near the apex. It seems reasonable that the first and second types of movement depend on actin filaments, while the third reflects unattached plastids in or near the pollen tube clear zone. Now that plastids can be specifically imaged in the pollen tube, it should be possible to characterize and understand their motility.

Golgi bodies and vesicles
The localization of Golgi bodies in pollen tubes has been analyzed by different methods, including the use of antibodies against specific proteins (Wei et al. 2005), the use of fluorescent dyes (Moscatelli et al. 2007), or the transformation of pollen with chimeric genes expressing Golgi proteins fused with fluorescent markers (Cheung and Wu 2007). To date, the picture that emerges describes the Golgi apparatus as widespread in the cytoplasm of pollen tubes with the exception of the apical region. As in other plant cells, it is likely that Golgi bodies are mobile in the cytoplasm. Motility of Golgi bodies in somatic cells was demonstrated by trans- forming plants with Golgi proteins fused to fluorescent markers (Nebenführ et al. 1999). The Golgi bodies actively move along specific tracks, most likely represented by actin filaments. The movement of Golgi bodies was defined as "stop- and-go" because those structures moved actively but also frequently stopped, possibly coinciding with the communication with the endoplasmic reticulum. It is likely that Golgi bodies are physically connected with the endoplasmic reticulum even during the stationary phase suggesting that Golgi bodies might be important for remodeling of the endoplasmic reticulum (Sparkes et al. 2011). The differential movement of Golgi bodies might be regulated by the organization of the cytoskeleton. For example, in root epidermal cells, Golgi bodies have distinct patterns of movement in relation to actin organization; Golgi bodies move rapidly along actin filament bundles but showed a non-directional movement when associated with fine filamen- tous actin (Akkerman et al. 2011). This suggests that the structure of the actin tracks is important in determining the movement of Golgi bodies. When this concept is applied to the pollen tube, the actin filament bundles along the tube's longitudinal axis might support Golgi trafficking at high speed; when Golgi bodies come upon the actin fringe, changes in actin structure might decelerate the movement of Golgi bodies and eventually reverse the motion. In addition to actin filaments, Golgi movement might be also regulated by the microtubular cytoskeleton. The Golgi is one of the few membrane-bound structures that have been demonstrated to be associated in plants with microtubule motors (Lu et al. 2005; Wei et al. 2009). This association is possibly required for the "stop" phase of Golgi body motility or for the release of secretory vesicles. The dynamic association with microtubules might also be necessary to localize the Golgi specifically in distinct cell areas where secretion of Golgi material is required. An interesting working hypothesis relates the stop (pausing) phase with the release of cellulose synthase and the organization of cellulose microfibrils but this remains to be confirmed (Zhong et al. 2002; Zhang et al. 2010b; Zhu and Dixit 2011). The association of kinesins with Golgi membranes is also suggested for pollen tubes in several studies. For example, a protein immunologically and sequen- tially related to Golgi kinesins was identified in N. tabacum pollen tubes and partially localized in association with Golgi bodies (Wei et al. 2005). Furthermore, Golgi-derived vesicles isolated from pollen tubes were observed to move along microtubules (Romagnoli et al. 2007) and proteins immuno- logically related to kinesin were found in association with partially-purified Golgi vesicles of Corylus avellana pollen (Liu et al. 1994).

VESICLE TRAFFICKING IN THE TIP IN RELATION TO POLLEN TUBE GROWTH

While the study of Golgi movement is relatively recent, the first analysis of the transport and accumulation of Golgi vesicles was the pioneering work of Picton and Steer (1981), who estimated the production rate of secretory vesicles and the quantity of vesicles produced by each Golgi body in T. virginiana pollen tubes. In that work, the authors showed that the integrity of actin filaments is necessary for the undiminished transport of vesicles. The content of vesicles was suggested to be pectic polysaccharides (Heslop-Harrison and Heslop-Harrison 1992); vesicles also were characterized as positive for arabinofuranosyl residues (Anderson et al. 1987), which is consistent with pectin. A detailed analysis by electron microscopy with freeze-fixation and freeze substitution techniques showed that secretory vesicles accumulate at the apex of L. longiflorum pollen tubes form the so-called inverted cone-shaped area (Lancelle and Hepler 1992). The accumulation of vesicles at the apex also requires integrity of the membrane systems (especially of Golgi) as demonstrated by the use of brefeldin A, which inhibited the growth of pollen tubes and the secretion of vesicles in the tip causing the disappearance of Golgi bodies and the formation of atypical membrane structures (Rutten and Knuiman 1993; Ueda et al. 1996). Generally, it is supposed that the amount of vesicles at the apex is higher than actually required to promote the growth of pollen tubes. By inhibiting the production of vesicles with brefeldin A, pollen tubes of N. tabacum and Petunia hybrida still grow for 10–25 min suggesting that the production rate exceeds the actual demand secretory vesicles (Geitmann et al. 1996a). Perhaps, the excess of vesicles reflects the requirement for a "buffer" of vesicles that should move toward the apical plasma membrane and fuse simply by diffusion. In any case, the number of secretory vesicles is apparently not limiting for growth. The accumulation of secretory vesicles at the pollen tube apex requires the integrity of the cytoskeleton (especially of actin filaments) (Gibbon et al. 1999; Vidali et al. 2001). Thus, the most plausible model suggests that Golgi vesicles, once produced by Golgi bodies, move along actin filaments and accumulate in the apical region where they either can fuse directly or can be recycled a number of times before fusing with the apical plasma membrane. The pattern of vesicle movement is somehow different from that of other organelles because vesicles are likely to move forward and backward and they also accumulate in the apex. When they are visualized by fluorescent dyes (Parton et al. 2001) or by GFP-tagged proteins (de Graaf et al. 2005), vesicle-like structures can be seen to move in the shank toward the apex, after that they accumulate consistently in the tip giving rise to the inverted cone. Apart from accumulating in the apex, vesicles also move backward for recycling or for transporting molecules to be degraded. This backward movement usually takes place in the center of pollen tubes. The classic model of transport and fusion of vesicles at the apex was based on the assumption that the point of maximum fusion rate coincides with the extreme tip of pollen tubes. Any process of vesicular recycling could occur in immediately adjacent or even relatively distant regions (Moscatelli et al. 2007). An alternative view suggests that secretory vesicles do not fuse exactly at the extreme apex but at the shoulders (sub-apex) so that the extreme tip is the starting point for endocytotic events (Bove et al. 2008). The pollen tube sub-apex as the fusion site of secretory vesicles is also supported by the localization there in A. thaliana of the syntaxin, SYP124, a component of a SNARE complex implicated in exocytosis (Silva et al. 2010). Mathematical modeling of vesicle flow suggests that the sub-apical actin fringe focuses vesicles so that they fuse with the plasma membrane closer to the apical edge of the fringe than the very apex of the cell (Kroeger et al. 2009). The focusing step exerted by the actin fringe might be active (i.e., propulsion is exerted by myosin molecules). Myosins have been detected in the apical and sub-apical regions of pollen tubes but their association with the actin fringe is hypothetical. In the root hairs of A. thaliana, a myosin XIK accumulates at the hair tip and is apparently involved in the dynamic organization of the actin cytoskeleton rather than directly powering vesicle or endoplasmic reticulum movement (Park and Nebenfuhr 2013). Nevertheless, the actin fringe is a characteristic structure of pollen tubes and it has not, to our knowledge, been detected in other cell types. Because root hairs and pollen tubes although both tip-growing cells are functionally distinct, we can speculate that myosins at the pollen tube tip might be involved in the organization of the actin cytoskeleton as well as directly powering vesicle delivery. Confirmation of this hypothesis requires in depth localization of myosins and functional analysis. Once vesicles have abandoned the actin fringe, what occurs in the extreme apex is perhaps dependent on vesicle diffusion: either a vesicle fuses with the plasma membrane or it is recycled in the next round or it is routed to the retrograde flow. Vesicle trafficking is

necessarily related to pollen tube growth. Secretory vesicles transport new plasma membrane and cell wall components that are required for tube elongation. The vesicle membrane likely contains some of the proteins and lipids that are to be inserted in the apical plasma membrane, while vesicles carry polysaccharides that will constitute the primary cell wall of pollen tubes. Consequently, the trafficking of apical vesicles has often been studied in relation to pollen tube growth and to cell wall deposition; this allowed researchers to build a model in which secretory vesicles are involved in the transport of proteins and membranes required for the synthesis and assembly of the cell wall (Hepler and Winship 2015). In this context, two types of molecular components (pectins and the polysaccharide- synthetizing enzymes) can be used to trace the movement of apical vesicles. Several observations suggest that pectins and enzymes are transported by secretory vesicles moving along actin filaments and accumulating in the apical region (Geitmann et al. 1996b; Jauh and Lord 1996). The journey of pectins starts in the Golgi (although these data have not yet been validated in pollen tubes) where they are produced in the methyl-esterified form. Pectins are transported within secretory vesicles moving in the shank and accumulating at the apex where they fuse with the plasma membrane. Secretion of pectins occurs in the apical region as demonstrated by the presence of methyl-esterified pectins at the pollen tube apex (Li et al. 1994) and by labeling pectins with fluorescent markers such as propidium iodide (Rounds et al. 2011a); after being incorporated in the cell wall, pectins are subsequently converted into the acid form by pectin methyl-esterase (Bosch and Hepler 2005). Endocytotic events are undoubtedly important for removing inhibitors of pectin methyl-esterase which then allows the conversion of methyl-esterified pectins into acidic pectins (Rockel et al. 2008). Membrane-associated polysaccharide-synthesizing en- zymes are represented by cellulose synthase and callose synthase. It is not known whether the same secretory vesicles carry pectins and enzymes for the synthesis of cellulose and callose. It is also not known where both enzymes are secreted but several indications suggest that they are secreted at the apex. Like most proteins to be secreted, callose synthase and cellulose synthase are presumably produced in the endoplasmic reticulum, exported to the Golgi bodies and then packaged into secretory vesicles. The association of callose synthase with Golgi membranes has been demonstrated by immunological and biochemical data, which indicate the progressive and consistent accumulation of the enzyme in the apical plasma membrane of N. tabacum pollen tubes (Brownfield et al. 2007, 2008; Cai et al. 2011). Inhibitors of actin dynamics and membrane trafficking significantly affect the deposition pattern of callose synthase indicating that the enzyme is transported within secretory vesicles actively moving along actin filaments. Movement and activation of the enzyme are also likely to depend on microtubules because microtubule drugs affect the distribution of callose synthase in the shank, far from the tip, indicating that secretion is not confined to the apex (Cai et al. 2011). Cellulose synthase-like proteins have been visualized after fusion with fluorescent markers in A. thaliana (Wang et al. 2011b) and by using heterologous antibodies in N. tabacum pollen tubes (Cai et al. 2011). As cellulose is detected at the apex, it is likely that cellulose synthases are active as soon as they are inserted. Distribution of the enzyme is restricted to the apex and sub-apex suggesting that it might be recovered by endocytosis and eventually recycled (Cai et al. 2011; Wang et al. 2011b). The pattern of cellulose synthase is perturbed by inhibitors of membrane trafficking and actin dynamics (Cai et al. 2011); consequently, cellulose synthase like callose synthase appears to be transported within vesicles that move along actin filaments. The involvement of microtubules in the movement of cellulose synthase is unclear in pollen tubes but it is widely recognized in other plant cells (Lei et al. 2014). The characterization of the relationship between cellulose synthase and microtubules in somatic cells enabled the discovery of a membranous compartment that is said to represent intermediates in the process of secretion or recycling (Crowell et al. 2009; Gutierrez et al. 2009). The movement of secretory vesicles towards the apex and their fusion with the plasma membrane is the basis for the insertion of enzymes in the apical membrane and for the secretion of new pectins. This process is synchronized with the growth rate of pollen tubes. Therefore, it is reasonable that this process (from movement to fusion) is accurately regulated by a complex network of molecules. Some of these regulatory events are discussed in the next section.

REGULATION OF ORGANELLE AND VESICLE TRAFFICKING

Trafficking of vesicles culminates in their fusion with distinct subcellular compartments such as the plasma membrane, the Golgi, or the endosomal system. Selective fusion of mem- branes determines and maintains the compartmentalization of pollen tubes; therefore, the regulation of vesicle transport also requires vesicles be delivered to and fuse with the correct membrane compartment. The specificity of this process requires the presence and activity of several factors working at the level of both vesicle and target membranes. The regulation of vesicle trafficking is usually achieved by using molecular switches belonging to the class of small GTPases. In pollen tubes, two small GTPases of the Rab subfamily are involved in the regulation of vesicle trafficking. The first is Rab11b (and its homologue RabA4d), which is localized in the tube apex of tobacco, probably in association with exocytotic vesicles (de Graaf et al. 2005). Analysis of Rab11b mutants suggested that it is involved in the polar organization of pollen tubes, possibly by promoting the delivery of vesicle-transported molecules involved in cell polarity. The second molecular switch, Rab2, regulates the trafficking between endoplasmic reticulum and Golgi in N. tabacum pollen tubes. Rab2 may be of particular importance during the "stop" or "pausing" phase of Golgi bodies along the endoplasmic reticulum (Cheung et al. 2002). Additionally, small GTPases of the Rho of plants (ROP) subfamily are found in the pollen tube apex and they are likely involved in vesicle trafficking by spatially organizing the actin cytoskeleton (Gu et al. 2003; Lin et al. 2015). ROP GTPases also interact with receptor-like kinases and mediate the transduction of extracellular signals into cellular processes to target pollen tube growth to the female gametophyte (Zou et al. 2011). By means of the activity of interacting proteins (like RIC3 and RIC4) (Gu et al. 2005), ROP proteins might contribute to interface the actin cytoskeleton with external signals, thereby synchronizing pollen tube growth with organelle transport. ROP proteins also interact with exocyst-binding proteins (like RIP1 and ICR1) in A. thaliana pollen tubes (Li et al. 2008)   suggesting that ROP proteins mediate the docking and fusion of secretory vesicles in cooperation with the exocyst. The latter is a complex of eight proteins that targets vesicles to the plasma membrane for fusion. One ortholog of the subunit Sec8 has been identified in the pollen tube of Arabidopsis and shown to be critical for tube elongation (Cole et al. 2005). The RIP/ICR1 family also contains members (such as RIP3) that have been demonstrated to interact with the active form of ROP. Interestingly, RIP3 localizes in association with microtubules and interacts with the kinesin-13 (Mucha et al. 2010). Generally, this suggests that ROP proteins, through effectors such as RIP3, also regulate the dynamics of microtubules in addition to that of actin filaments. ROP proteins are therefore candidates for central regulators of pollen tubes by networking actin and microtubule dynamics to vesicle fusion and to pollen tube growth. Another subfamily of small molecular switches that regulate vesicle trafficking are the Arf GTPases. In pollen tubes, evidence for Arf-like proteins is indirect; although neither Arf proteins themselves nor the inactivating Arf-GAPs have been described in pollen (Song et al. 2006), an Arf-GEF, appears to be involved in endosome trafficking (Liao et al. 2010). The selective fusion of membranes is usually determined by proteins of the SNARE family. Members of this family are likely to be present in pollen but only a few studies exist on pollen SNAREs. One such study reports that Syp2 and Syp4, which are members of the syntaxin family, participate in vesicle trafficking between vacuoles and the trans-Golgi-network (Sanderfoot et al. 2001). Another syntaxin-like protein (A. thaliana SYP124) is likely to mediate the fusion of exocytotic vesicles at the tube apex (Silva et al. 2010). Members of the SNARE family are also represented by vesicle-associated membrane protein 7 (VAMP7) proteins, which mediate membrane fusion; homologs have been also identified in pollen tubes in association with the inverted cone of transport vesicles (Guo and McCubbin 2012).

PROSPECTS

When we show growing pollen tubes during practical courses in plant biology, the general comment of students is often a mixture of curiosity, astonishment, and wonder. The students' reaction to the vision of organelle trafficking, which can be easily observed in a pollen tube, reminds us how beautiful is the spectacle of life and that "movement" is a perfect synonym for "life". Although this phenomenon is easily observable in many details, we unfortunately lack information to decipher and understand this process. Organelle trafficking is a complex series of events, which requires the interplay between several molecules. An exhaustive list is hard to articulate; nevertheless, we can identify some specific points whose clarification will shed light on the entire process. First, the interplay between actin filaments and

microtubules needs to be elucidated to a greater extent. This does not simply require us to determine the relative distribution of both cytoskeletal systems but to depict how their cross-talk affects organelle and vesicle movement. We also need to extend knowledge of the molecular interplay to the functional cooperation between motor proteins. Do microtubules effectively stop or slow-down organelles and vesicles at specific cell sites? Are kinesins involved in this process (Cai and Cresti 2012)? Both in vitro motility assays and the use of mutants for genes coding pollen- expressed motor proteins will be valuable to elucidate this process. Another important topic to dissect further is the role of the actin fringe. Although information is accumulating on this structure, we need to realize how, or even if, it acts to exclude large organelles from the tip. Does the actin fringe cooperate with myosin in the exclusion process? What is the relation between the extension of actin fringe, the pH gradient, and the alkaline band, and organelle movement? Finally, how do external signals interface with the mechanism of organelle trafficking? The elucidation of these and other research points will not only contribute to our understanding of this cell model but, by extending information to other cells, we can also appreciate how organelle movement is fully optimized for the several cyto-physiological events of plant cells.

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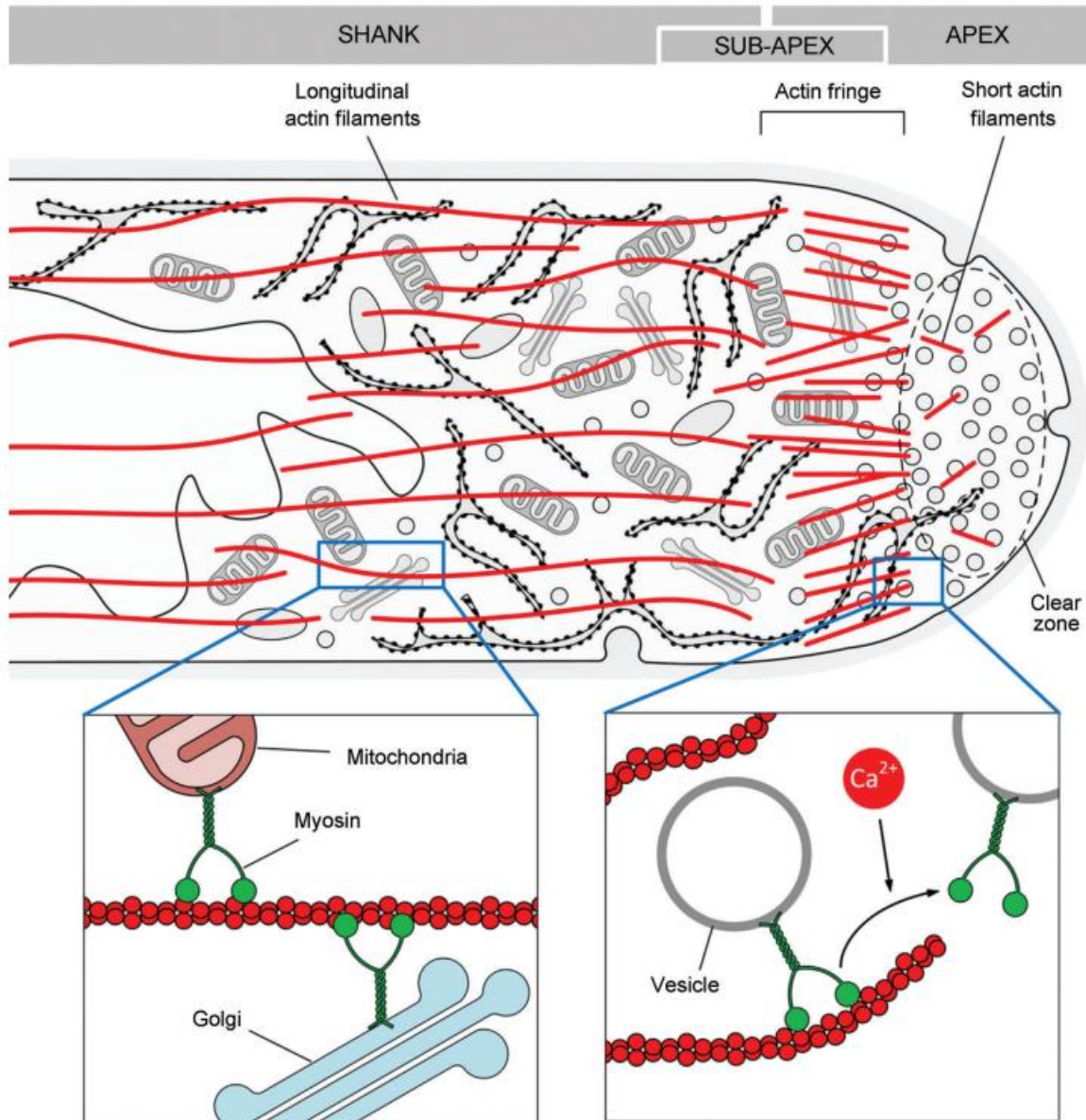
Figure 1. Arrangement of actin filaments in pollen tubes and activity of myosin

Three different organizations of actin filaments are known to exist in pollen tubes: longitudinal actin bundles, which run along the entire length of pollen tubes, the so-called "actin fringe" (i.e., a kind of actin collar located approximately in the pollen tube sub- apex) and short actin filaments scattered at the pollen tube apex. As indicated in the lower left panel, organelles actively move along actin bundles driven by myosin. Secretory vesicles (lower right panel) accumulate mainly in the apical region whereas larger organelles are excluded, possibly because of a filtering action of the fringe. Vesicles might be driven directly via myosin or might move indirectly through bulk flow or diffusion. The presence of a high calcium concentration at the apex might inactivate myosin and promote actin disassembly, allowing vesicles a free zone within the apex.
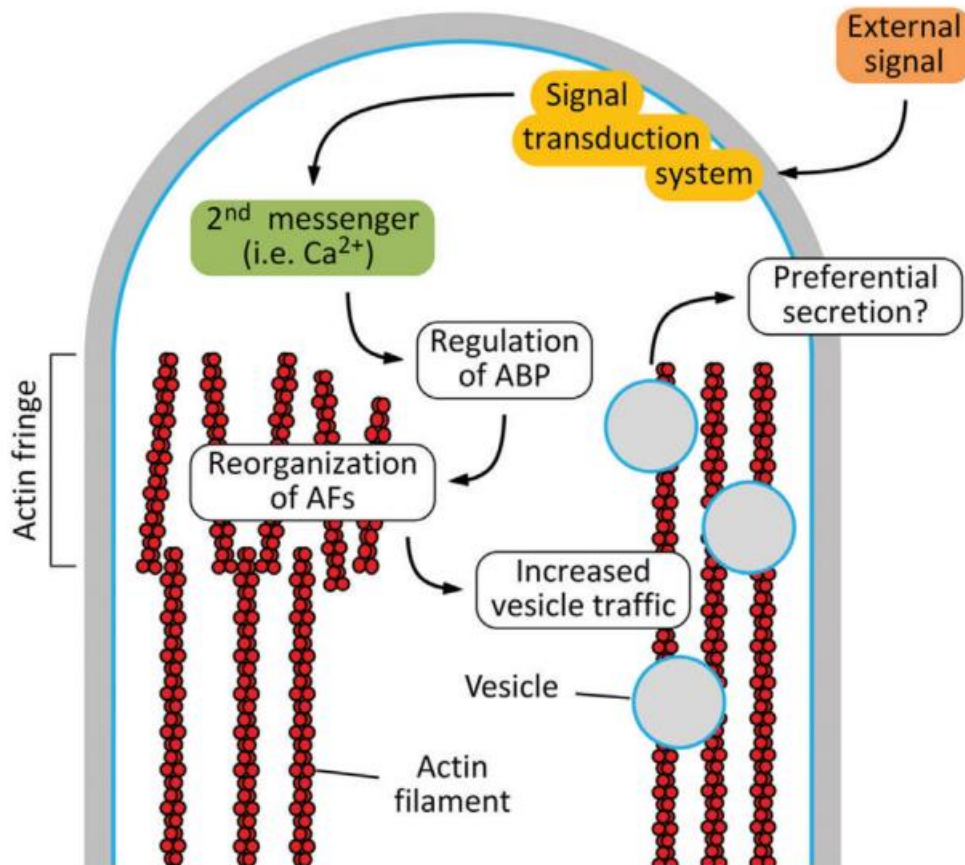
Figure 2. Simplified hypothesis of the relationship between vesicular trafficking and growth direction of pollen tubes

External cues that regulate the growth direction are perceived by the signal transduction system located in the plasma membrane and including the receptor system, the different Rho and Rac proteins, phospholipases, and other molecules. One of the consequences of a directional stimulus could be an asymmetric calcium distribution. By regulating the activity of actin-binding proteins (ABP), the signal transduction mechanism induces a changed organization of actin filaments (both for the extension of actin fringe and for the elongation of actin bundles). The result would be a differential movement of secretory vesicles that accumulate in the apical region of pollen tubes where the signal was received. This could lead to the increased growth of that region resulting in the change of growth direction.
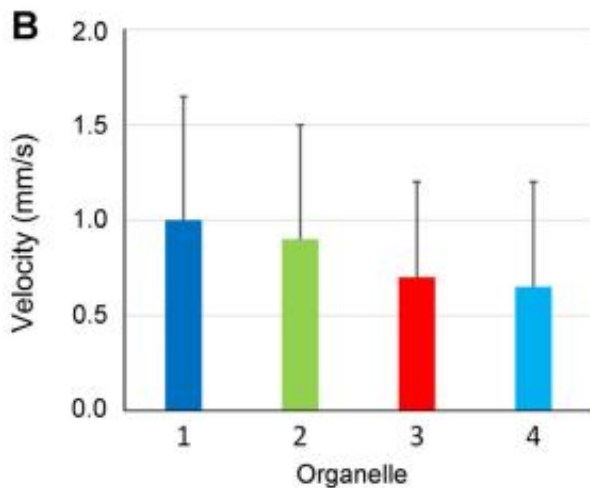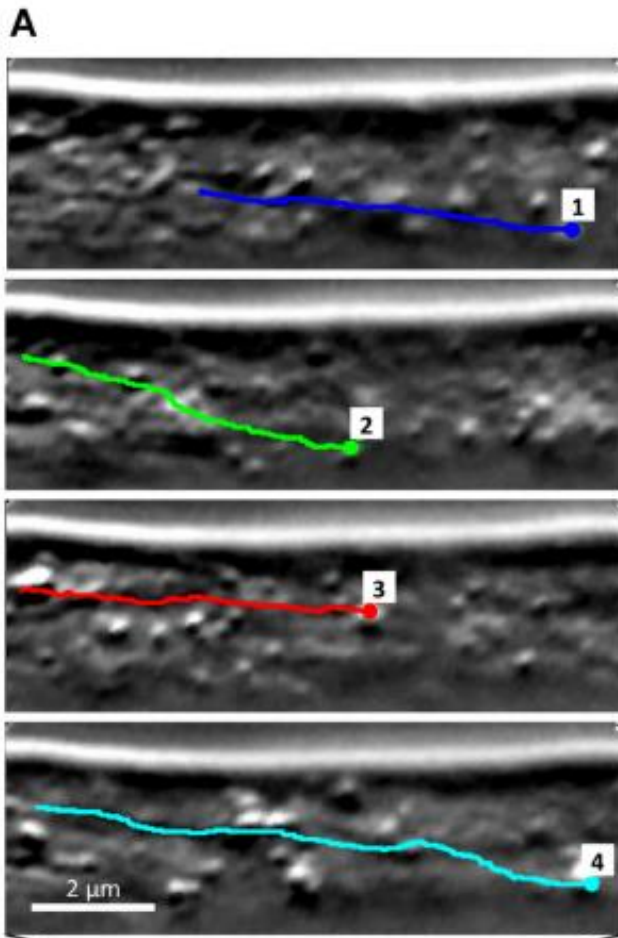
Figure 3. Monitoring the trafficking of organelles in pollen tubes
(A) Pathways followed by four organelles in a Nicotiana tabacum pollen tube. Growing pollen tubes were imaged for 10–20 s, with frames acquired every 0.5 s. Image sequences were analyzed by ImageJ (Manual Tracking plugin) and the trajectories of four selected organelles are overlain on the initial frame. The area imaged is schematically indicated in the sketch at the bottom. (B) For each of the four organelles, absolute velocities were obtained for each interval and averaged over the entire trajectory.