Rational Analyses of Organelle Trajectories in Tobacco Pollen Tubes Reveal Characteristics of the Actomyosin Cytoskeleton

Anna H. N. de Win, Elisabeth S. Pierson, and Jan Derksen
Laboratory of Plant Cell Biology, Department of Experimental Botany, Graduate School of Plant Science, Catholic University of Nijmegen, Nijmegen, The Netherlands

ABSTRACT To gain insight into the characteristics of organelle movement and the underlying actomyosin motility system in tobacco pollen tubes, we collected data points representing sequential organelle positions in control and cytochalasin-treated cells, and in a sample of extruded cytoplasm. These data were utilized to reconstruct ~900 tracks, representing individual organelle movements, and to produce a quantitative analysis of the movement properties, supported by statistical tests. Each reconstructed track appeared to be unique and to show irregularities in velocity and direction of movement. The regularity quotient was near 2 at the tip and above 3 elsewhere in the cell, indicating that movement is more vectorial in the tube area. Similarly, the progressiveness ratio showed that there were relatively more straight trajectories in the tube region than at the tip. Consistent with these data, arithmetical dissection revealed a high degree of randomlike movement in the apex, lanes with tip-directed movement along the flanks, and grain-directed movement in the center of the tube. Intercalated lanes with bidirectional movement had lower organelle velocity, suggesting that steric hindrance plays a role. The results from the movement analysis indicate that the axial arrangement of the actin filaments and performance of the actomyosin system increases from tip to base, and that the opposite polarity of the actin filaments in the peripheral (+-ends of acting filaments toward the tip) versus the central cytoplasm (+-ends of actin filaments toward to the grain) is installed within a few minutes in these tip-growing cells.

INTRODUCTION

Pollen tubes, the carriers of the male gametes in plants, are cylindrical cells with a dense cytoplasm and remarkably rapid growth restricted to the tip (for reviews see Steer and Steer, 1989; Mascarenhas, 1993; Derksen et al., 1995b; Taylor and Hepler, 1997). The distribution of organelles from the tip to the base of the cell is not uniform, but it is rather symmetrical when observed along the transverse radius (for tobacco: Derksen et al., 1995a). Pollen tubes growing in vitro have vigorous cytoplasmic streaming, and therefore are often used as model cells to study the cytoskeleton. Early reports depicted the cytoplasmic streaming as a reverse fountainlike bulk flow of particles (Iwanami, 1956, 1959). Later works addressing the structure and biochemical nature of cytoskeletal elements, the effect of selective inhibitors of the cytoskeleton, and in vitro motility assays demonstrated unequivocally that pollen tubes, like other plants cells, exclusively utilize an actomyosin-based system to generate the movement of large organelles, i.e., dictyosomes, mitochondria, and plastids (Lancelle and Hepler, 1988; Kohno et al., 1990; Miller et al., 1995; Yokota et al., 1995; for reviews see Pierson and Cresti, 1992; Steer and Steer, 1989; Mascarenhas, 1993; Derksen et al., 1995b; this study). With the observation of single organelles by video microscopy (Heslop-Harrison and Heslop-Harrison, 1987, 1988, 1990, 1994; Pierson et al., 1990), the simple consensus that organelles move smoothly back and forth in the pollen tube along rigid tracks of axially oriented actin filaments started to be questioned, but was not tested. To address this problem technical conditions had to be improved so that it became possible to track a large number of organelles in the cytoplasm of pollen tubes.

With the introduction of a semiautomated procedure (de Win et al., 1998), we were able to collect extensive data on sequential organelle position in tobacco pollen tubes. In this study we have utilized these data to reconstruct organelle tracks and to produce qualitative and quantitative analyses of movement patterns in various regions of the cell. The following quantitative analysis methods have been applied: the *regularity quotient* (Q_r; Jarosch, 1956), the *progressiveness ratio* (*P;* Overstreet et al., 1979), a new method called arithmetical dissection, and statistical tests. The characteristics of the movement patterns have been employed to formulate guidelines by which the underlying actomyosin motility system operates in living pollen tubes.

MATERIALS AND METHODS

Biological material

Pollen of *Nicotiana tabacum* L. (tobacco) cv. Samsun was collected from plants grown under greenhouse conditions, dried, and stored at −20°C until use. Pollen was cultured at 28°C for 1.5–2.5 h on 3-mm punches of sterilized Visking dialysis tubing (Serva, Heidelberg, Germany) placed on a medium containing 10% sucrose and 0.01% boric acid solidified with 2% agarose.

Received for publication 24 April 1998 and in final form 12 December 1998.

Address reprint requests to Dr. E. S. Pierson, Laboratory of Plant Cell Biology, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands. Tel.: +31-(0)24-3652777; Fax: +31-(0)24-3652490; E-mail: epierson@sci.kun.nl.

0006-3495/99/03/1648/11 $2.00

Cytoskeletal inhibitors

We added either actin filament inhibitors (10^{-5}, 2.0×10^{-5}, 10^{-4}, and 2.0×10^{-4} M cytochalasin B, or 2.0×10^{-5}, or 10^{-5} M cytochalasin D, final concentration in culture medium), or a microtubule inhibitor (10^{-3}, 10^{-2}, and 5.0×10^{-2} M colchicine, final concentration in culture medium) to samples of growing tobacco pollen tubes showing normal cytoplasmic streaming. Video images of organelle movements, recorded before and after drug treatment according to the set-up described in de Win et al. (1998), were used for qualitative analysis. In addition, quantitative analyses were performed on 47 organelles in a pollen tube treated with 2.0×10^{-5} M cytochalasin D.

Data acquisition

In a previous paper (de Win et al., 1998), we provided details and critical notes on the digital procedure employed to collect data on the sequential positions of organelles in pollen tubes viewed with a video microscope. Briefly, the procedure was used to acquire ~15,000 organelle positions from 900 organelles from time-lapse images (0.40-s time interval) in three growing control pollen tubes (length up to 200 μm, diameter between 8.4 and 10.4 μm), one cytochalasin-treated cell, and a sample of *free organelles* forced out of pollen tubes by pressure. We sampled the positions of *all* trackable particles with a spherical appearance and a diameter between 0.2 and 0.6 μm within series of microscopic images until the required number of organelles was reached (see next paragraph on sample size). This sampling method includes virtually all organelles participating in cytoplasmic streaming, but only excludes extremely large or small particles, like tubular endoplasmic reticulum, most vacuoles, and Golgi vesicles, the only particles that accumulate in the very tip and are exocytosed. In pollen tubes, tip growth and organelle movement are colchicine-insensitive, but cytochalasin-sensitive (Steer and Steer, 1989; Pierson and Cresti, 1992; this study). Although Golgi vesicles have been reported to be associated with an immunoreactive homolog of kinesin (Cai et al., 1996), there is no evidence, for example from in vitro motility assays, that these organelles, or any other particles in pollen tubes, are translocated by a microtubule-dependent system.

All data on the organelle positions in pollen tubes are shown in a cell-defined coordinate system in which the long axis of the pollen tubes (i.e., the middle line in the median plane of these cylindrical cells) was defined as the x axis, and the tangent through the extreme pollen tube tip was defined as the y axis. Hence, x and y axes are orthogonal. Except for minor adjustments, organelle tracks were segmented into sequences divided over 6-μm-wide regions along the long axis of the tubes (see layout in Fig. 7). These sequences are called trajectories throughout the text. Trajectories shorter than 2 s or with time gaps, which essentially arose from a back-and-forth movement of an organelle over two adjacent 6-μm-wide regions, were excluded from further analysis. Each displacement had a distance parameter (Dist); that is, the distance between two consecutive positions, to which a velocity parameter (Vel) could be attributed. The average of Dist or Vel over the duration of a trajectory was denoted as $\langle \text{parameter} \rangle$. An average over trajectories in a subset was denoted as $\overline{\text{parameter}}$. Population features were calculated with (time)-averaged parameter(s) per trajectory.

Positional data were analyzed with the modules BASE, GRAPH, and STAT (version 6.07) of SAS (Statistical Analysis System; SAS Institute Inc., Cary, NC). SAS provides a variety of standard procedures for scientific analysis, and additional possibilities to calculate new features or customize the standard output. Either the programs SAS or Microcal origin (version 3.5; Northampton, MA) was used to make plots.

Sample size and timing

To determine the minimum number (N_1, N_2) of organelles necessary for a representative and uniform sampling of the organelle population, we chose a difference (d) in the average mean velocity of 0.1 μm/s between two samples as a decisive criterion. This velocity is based on the size and displacements of organelles, and window of errors in the acquisition procedure (de Win et al., 1998). The values for the variances (s_1, s_2) in the average mean velocity over the first five displacements of the first 50 organelles of a sample, and the value for the desired significance level Z ($Z\alpha = 1.96$ for 95% significance) were entered in Eqs. 1 and 2 (Mace, 1964):

$$N_1 = s_1(s_1 + s_2)(Z\alpha/d)^2 \quad (1)$$

$$N_2 = s_2(s_1 + s_2)(Z\alpha/d)^2 \quad (2)$$

When the variations in velocity were high, sometimes more than 150 organelles per sample were needed to meet the criteria. In the opposite case, when values for N were below 50, still an absolute threshold of at least 50 organelles was applied.

The moment data collection was initiated did not affect the analytical results (tests shown in de Win, 1997). We established this fact by performing Wilcoxon and median rank score tests on a subset of organelles already present in the plane of view at the beginning of the measurement, and another subset consisting of an equal number of organelles coming into view during the sampling. The parameters tested were the mean velocity over five displacements, the P and Q_r values (see below). None of these tests showed significant differences among the two subsets.

Rational data analyses

Regularity quotient

The mean-square displacement (MSD) (Qian et al., 1991), designed to monitor the general displacement in a population, reports on two components: pure diffusional movement (diffusion coefficient, D) and vectorial displacement (active movement or flow) with a velocity, v, at time points t (Eq. 3):

$$\text{MSD}_{(t)} = 4Dt + v^2t^2 \quad (3)$$

This proportionality was used by Jarosch (1956; see also Chang, 1981) to formulate a new parameter (Q_r), which can be applied to (small) samples measured over much shorter time periods than the true MSD. The Q_r is the ratio between the MSD during time interval t and the MSD during the double time interval t_2. The Q_r has a theoretical ideal value of 2 for Brownian movement, and 4 for uniform and linear movement. In contrast to the trajectories investigated by Jarosch (1956) in onion cells, the trajectories we collected in pollen tubes were not of equal duration and length. To compensate for this fact, MSDs for the distance over the standard time interval ($\langle \text{Dist}^2 \rangle$) and for the distance over the double time interval ($\langle \text{Dist}_d^2 \rangle$) were first determined for each trajectory. Next, the average of all trajectories was taken in order to obtain a Q_r value that characterized a population (Eq. 4).

$$Q_r = \overline{\langle \text{Dist}_d^2 \rangle} / \overline{\langle \text{Dist}^2 \rangle} \quad (4)$$

Progressiveness ratio

The straightness of a trajectory can be described by (P), which is the ratio between the net distance between the last (x_e, y_e) and the first (x_0, y_0) position, and the total distance covered over all displacements (Σ Dist) (Overstreet et al., 1979; Eq. 5):

$$P = \sqrt{(x_e - x_0)^2 + (y_e - y_0)^2} / \sum \text{Dist} \quad (5)$$

Thus, $P = 1.0$ for an organelle moving in a straight line and it decreases as a trajectory becomes more wavelike. We employed predefined ranges for P to distinguish between *coiled* trajectories, for the range $0 < P < 0.5$, and straight or nearly straight trajectories, for the range $0.9 < P \leq 1.0$. The percentage of organelles belonging to each group was calculated to provide an indication of the consistency of the direction of movement.

Arithmetical dissection

To express the directionality and velocity of complex movements in relation to their orientation in the cell and the distance of the organelle from the tip, we introduced a new method, which we called arithmetical dissection. Time-sequences of organelle positions were routinely screened for an increase or decrease in the x and y coordinates in consecutive organelle positions. Movement was defined as *directed* when at least one of the coordinates (either x or y) consequently increased or decreased over a minimum of three consecutive displacements, with the contingency that a total minimal distance of 0.5 μm was also covered along one of the axes (Fig. 1). These criteria were set by taking into account the diameter of sampled organelles (~0.4 μm) and the average distance covered per displacement (0.22 μm). The threshold of 0.5 μm provides sufficient confidence that a translocation is real and not an artifact due to noise (see also de Win et al., 1998). Eight types of directed movement were defined according to the various combinations of x and/or y coordinate increase or decrease (see scheme in Fig. 1). Two other categories of movement were distinguished, but they belonged to the group of undirected movement: first, *nondirected* movement (type 9; Fig. 1), for cases in which three or more consecutive displacements in a sequence did not meet the preset conditions for directed movement, and second, *indefinable* movement (type 10; Fig. 1), for cases when only one or two consecutive displacements did not meet the preset conditions. Extensive examples of the arithmetical dissection procedure can be found in de Win (1997).

The relative contribution of any direction to the total movement in a (sub)population was analyzed with the *distance fraction*, defined as (Eq. 6):

$$\text{Distance fraction} = \overline{\sum \text{Dist}_{\text{type}} / \sum \text{Dist}} \qquad (6)$$

whereby $\Sigma\,\text{Dist}_{\text{type}}$ is the summed distance of all displacements in a trajectory with the specified movement type, and Σ Dist is the total of all the displacement distances in a trajectory. The total of all fractions was 1.0.

Statistical procedure

The Wilcoxon test for two classes, or the Kruskal-Wallis test for more than two classes, (both available in the NPAR1WAY procedure of SAS), were used to diagnose differences in magnitude and distribution among non-Gaussian-like (sub)populations of unequal size, i.e., the Q_r, P, Vel $\langle \text{Vel}_{\text{type}} \rangle$ (the mean velocities over sequences of displacements with a specific movement type within a trajectory), and distance fraction (Tables 1 and 2). In Table 1, data from a tip zone (for $x = [0–19]$ μm) were contrasted with data from a tube zone (for $x > 19$ μm). The boundary between these two zones was chosen on account of the patterns of the reconstructed tracks and cytological properties of pollen tubes (Derksen et al., 1995a, b). Separate tests on data from cytoplasmic lanes with uni or bidirectional movements obtained by arithmetical dissection were applied over 6-μm-wide regions (Table 2).

TABLE 1 Wilcoxon contrast tests for the distribution of features in two zones of the pollen tube long axis

Feature	Combined cells	Cell 1	Cell 2	Cell 3
Regularity quotient, Q_r	+			
Progressiveness ratio, $0.9 < P \leq 1.0$	+			
Progressiveness ratio, $0 < P < 0.5$	+			
Distance fraction, type 1		−	−	+
Distance fraction, type 5		+	−	−
Distance fraction, type 9	+			
$\langle \text{Vel}_{\text{type 1}} \rangle$, total region		+	+	+
$\langle \text{Vel}_{\text{type 1}} \rangle$, unidirectional lanes		+	+	+
$\langle \text{Vel}_{\text{type 1}} \rangle$, bidirectional lanes		−	+	−
$\langle \text{Vel}_{\text{type 5}} \rangle$, total region		+	+	−
$\langle \text{Vel}_{\text{type 5}} \rangle$, unidirectional lanes		+	+	−
$\langle \text{Vel}_{\text{type 5}} \rangle$, bidirectional lanes		+	+	−

The values of the tip zone (between 0 and 19 μm from the tip) were included in the first class and contrasted against the values of the tube zone (at a distance >19 μm from the tip). Tests were performed with the combined values of the three cells, when a Kruskal Wallis test indicated similarity among the cells. Otherwise, tests were performed for individual cells. Probability scores ≤0.05 were marked +, scores with $p > 0.05$ were marked −. Type 1 corresponds to tip-directed and type 5 to grain-directed movement according to Fig. 1.

RESULTS

Reconstructed tracks

The first impression given by the sum of reconstructed tracks was one of vectorial movement along the longitudinal axis in the main part of the pollen tube (antigrade along the

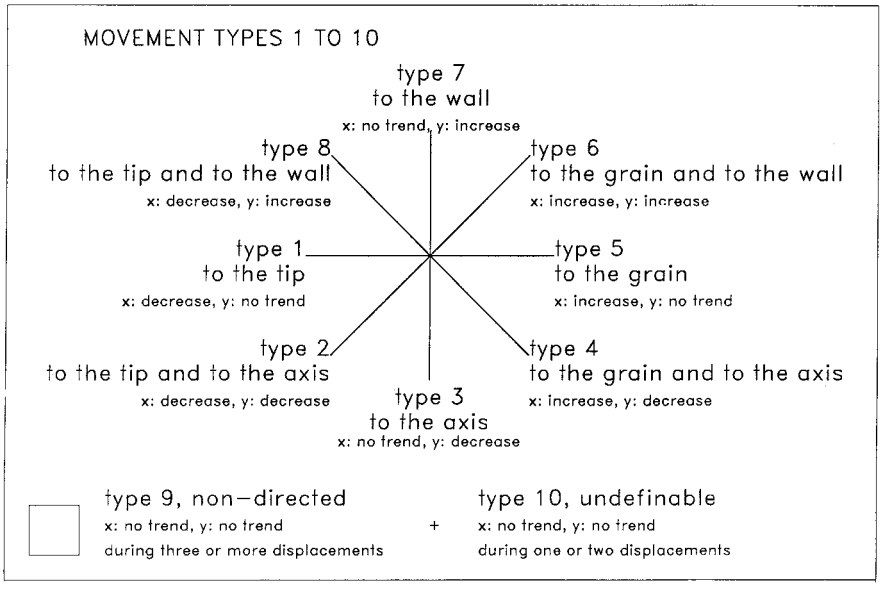

FIGURE 1 Arithmetical dissection of trajectories in types of movements. Eight possible combinations for directed movement are distinguished, depending on the combination of increase, decrease, or absence of trend in x- or y-coordinate, according to the definition given in the text. Displacements with no trend in both x and y direction belong either to the category of nondirected movement (type 9; three or more displacements with no trend in x and y direction; labeled as squares) or "indefinable movement" (one or two displacements with no trend in x and y direction; type 10; labeled as +). The scheme shown here is valid for trajectories above the x axis; trajectories under the x axis require a mirrored frame (not shown).

TABLE 2 Wilcoxon contrast tests for distributions of mean velocities, $\langle Vel_{type} \rangle$, in trajectories with movement type 1 and 5, applied to various lanes

	First class		Second class											
	TM	TL	TM	TL	Mid-position of the 6-μm-wide regions [μm from the tip]									
Cell 1					3.0	9.0	15	22	28	34		79	85	91
	1	all	5	all	*	−	−	+	+	+		+	+	+
	1	uni	5	uni		−	−	+	+	+		−	+	+
	1	bi	5	bi		*	−	−	−	−		−	−	−
	1	uni	1	bi		*	−	−	−	+		−	−	+
	5	uni	5	bi		*	−	+	+	+		*	+	−
Cell 2					3.0	8.5	15	22			69	76		
	1	all	5	all	*	−	−	+			+	*		
	1	uni	5	uni		*	−	+			+	*		
	1	bi	5	bi		−	*	−						
	5	uni	5	extra				+						
	5	bi	5	extra				−						
Cell 3					3.0	9.0	16	22				114	120	126
	1	all	5	all	*	−	−	+				−	*	+
	1	uni	5	uni		−	*	+						
	1	bi	5	bi		*						+	*	*
	1	uni	1	bi		*						+	+	*
	5	uni	5	bi		−								

Each test was done on two classes of data in 6-μm-wide regions, which differed either in their type of movement (TM) or in their type of lane (TL, defined by arithmetical dissection; unidirectional = uni; bidirectional = bi; all lanes = all; extra lanes = extra). Significant probability scores ($p \leq 0.05$) are marked +, and scores for which $p > 0.05$ are marked −. Tests in which one or both classes had less than five values are indicated by *.

flanks and retrograde in the center) and more disordered, randomlike movement in the cell tip. Screening of the tracks showed that each of the 900 tracks was unique (examples: the apex of cell 1 in Fig. 4 *a*, and all regions of cell 3 in Fig. 3 in de Win et al., 1998). Observations of the displacements in the graphical representation revealed variations in velocity between single organelles, also within the same region, as well as variations within the same track. As described for single trajectories by applying region-based curve analysis (de Win et al., 1997), some organelles showed straight tracks alternated with typical saltatory movement, particularly in parts of the cell with multidirectional movement. In a bulk flow situation it is improbable that single particles show sudden accelerations or decelerations, especially when these changes in velocity occur at different times and sites. To test this probability quantitatively for pollen tube organelles, we calculated the relative increase or decrease in velocity with respect to the next displacement within the same track. These measurements were carried out on all displacements, taken over a steady time-interval of 0.40 s, except, of course, for the last displacement within a track. The result was that on average 32% of all displacements showed at least a twofold increase or decrease in velocity, which is highly unlikely in a bulk flow situation. In general, tracks were longer and straighter in the center of the tube and away from the tip, and the length of the displacements indicated that the organelles moved more rapidly in the tube than in the tip (see data in the section on arithmetical dissection). Tracks were unevenly distributed over the length of the pollen tube. This fact probably reflects variations in organelle density, except for the extreme tip (0–2 μm from the cell edge) which is virtually completely occupied by Golgi vesicles that do not meet the sampling criteria. The free organelles, which are organelles extruded out of pollen tubes, showed cris-cross tracks (not shown). The average velocity of those organelles was much higher (2.62 μm/s) than that of organelles in the pollen tubes (0.52 μm/s).

Regularity quotient

Calculations of Q_r for the groups of trajectories in the 6-μm-wide regions and for a sample of free organelles ($Q_r = 1.82$) are reported in Fig. 2. The Q_r of the three pollen tubes indicated a rapid increase from a value close to 2.0 near the extreme tip, to above 3.2 at a distance exceeding 19 μm from the tip. The values for the tip zone ($x =$ [0–19 μm]) and the tube zone ($x > 19$ μm) were significantly different (Table 1).

Progressiveness ratio

The percentages of nearly straight and coiled trajectories, as defined by ranges for P (nearly straight: $0.9 < P \leq 1.0$;

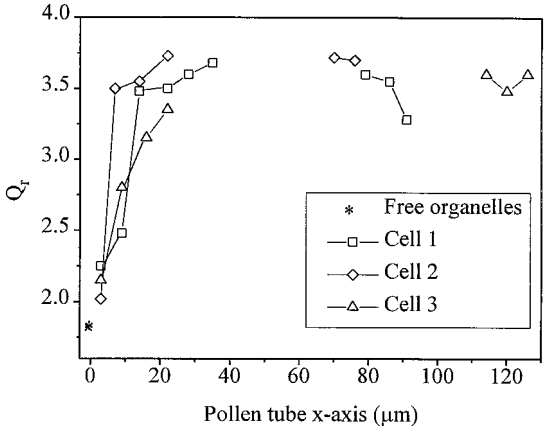

FIGURE 2 Regularity quotient (Q_r) for populations of trajectories in three pollen tubes. The Q_r values are plotted at the mid-position of the 6-μm-wide regions. The $*$ denotes the Q_r value for the sample of free organelles (plotted for practical reasons at $x = -0.5$ μm).

coiled: $0 < P < 0.5$), are shown in Fig. 3 for the 6-μm-wide regions and the sample of free organelles. The percentage of nearly straight trajectories (Fig. 3 *a*) was under 10% in the most apical region, but increased to ~40% at 25 μm from the tip, and became more variable in the regions of the tube farther from the tip. The percentage of coiled trajectories

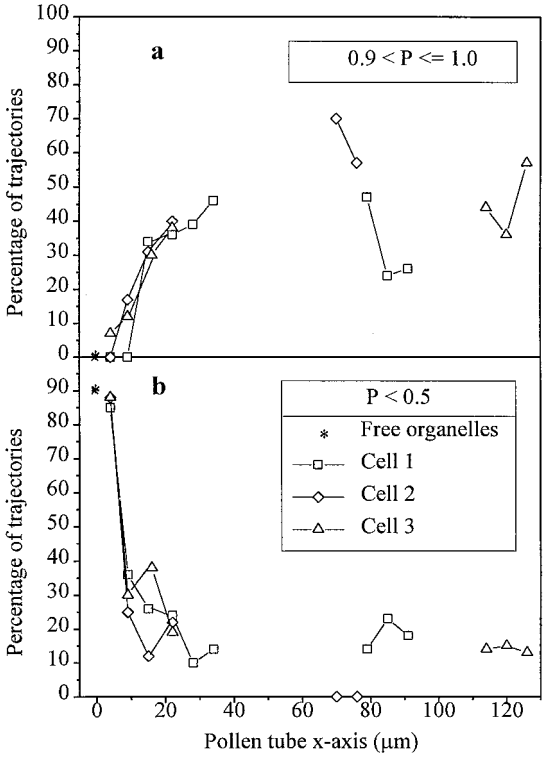

FIGURE 3 Percentage of trajectories in two classes of progressiveness ratios (P) in three pollen tubes. Percentages are plotted at the mid-position of the 6-μm-wide regions. (*a*) Percentage of nearly straight trajectories, for $0.9 < p \leq 1.0$. (*b*) Percentage of coiled trajectories, for $0 < P < 0.5$. The two $*$ represent the percentages for the sample of free organelles (plotted for practical reasons at $x = -0.5$ μm).

(Fig. 3 *b*) was high (75–85%) in the very tip, but quickly decreased to <25% behind the tip. No nearly straight trajectories are present among the free organelles, and ~90% of them had a P value < 0.5 ($*$ in Fig. 3, *a* and *b*). These descriptions were supported by statistical tests showing significant differences between the tip and tube zones (Table 1).

Arithmetical dissection and velocity analysis

Fig. 4 provides a representation of the movement types in cell 1, acquired by arithmetical dissection according to Fig. 1. Movements with at least one component directed to the tip (type 1, 2, and 8; Fig. 4 *b*), to the grain (type 4, 5, and 6; Fig. 4 *c*), and radial or nondirected and indefinable movements (type 3, 7, 9, and 10; Fig. 4 *d*) were presented separately.

In the chapter on reconstructed tracks we have seen that organelles in pollen tubes show sudden accelerations and decelerations. The grade of individuality of single tracks was further estimated by calculating the percentage of unique series of displacements. The screening was performed both on the movement type, as defined by arithmetical dissection, and the sequences of velocities after partition in 0.13 μm/s classes (class value corresponding to the error of spatial measurement, de Win et al., 1998). According to these criteria, a minimum of 73% (measured over 1.2 s) or 92% (over 2.0 s) of the series of displacements were unique in the three pollen tubes.

Calculation of the distance fractions for each movement type (Fig. 5) revealed that the major contributors were tip-directed and grain-directed movement (respectively Fig. 5, *a* and *b*) at a distance exceeding 19 μm from the tip, and nondirected movement in the apex (Fig. 5 *c*; Table 1). Movements with an oblique orientation compared to the axes (type 2, 4, 6, 8) and indefinable movements were rare in all regions (distance fractions < 0.05). There was hardly any radial movement, except in the most apical region where the distance fractions for these types of movement were between 0.05 and 0.10, which is significantly higher than in the other regions ($p = 0.0085$ for type 3 and $p = 0.0120$ for type 7). In the sample of free organelles, the distance fractions of the directed movement types were all lower than 0.2 (from 0.03 for type 8 to 0.16 for type 3; Fig. 5, *a* and *b*, for type 1 and 5). In this sample, nondirected movements contributed to a fraction of 0.29 of the total movement (Fig. 5 *c*) and indefinable movements to 0.10.

As shown in Fig. 6, a distinction could be made between lanes located preferentially in the periphery that had tip-directed types of movements (type 1, 2, and 8), and other lanes located in the center of the cell that had grain-directed types of movements (type 4–6). Lanes with movements that tended to move either to the tip or to the grain (unidirectional lanes) were interspersed with lanes having bidirectional movement (Fig. 6; see also deviations). No lanes were detected in the most apical region of the cells.

When entire 6-μm-wide regions were considered, the average mean velocity for tip- or grain-directed movements, re-

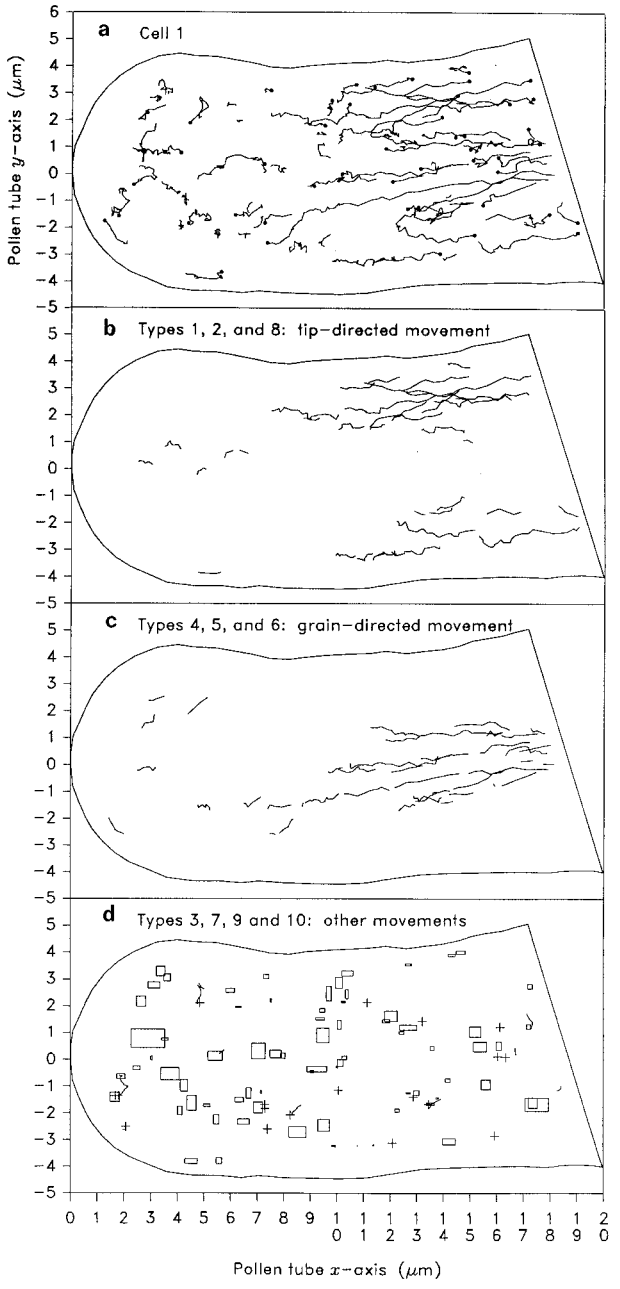

FIGURE 4 Reconstructed tracks, and trajectories classified by arithmetical dissection in movement types (Fig. 1). Shown are data on 81 organelles collected from a sequence of 39.6 s, in the apex of cell 1. (*a*) Reconstructed trajectories. Dots indicate the beginning of tracks showing directional movement. (*b*) Compilation of parts of trajectories with a component directed to the tip (types 1, 2, and 8). (*c*) Compilation of parts of trajectories with a component directed to the grain (types 4–6). (*d*) Compilation of the remaining types of movement. Parts of trajectories with radial movement (types 3 and 7) are drawn as lines, parts with nondirected movement (type 9) are outlined by squares, and parts of indefinable movements (type 10) are labeled with a +.

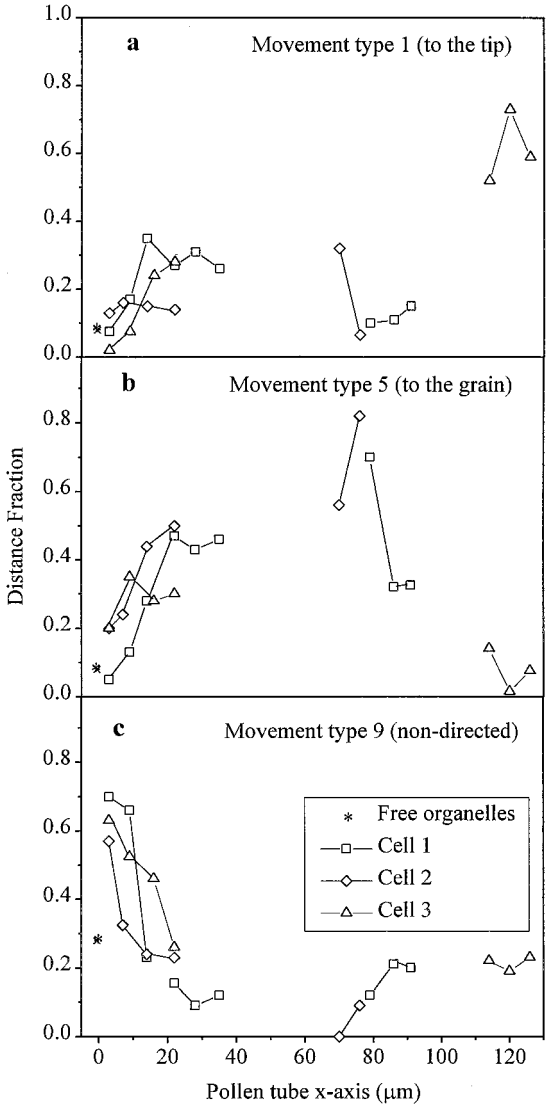

FIGURE 5 Distance fractions of the predominant movement types in three pollen tubes. Distance fractions are plotted at the mid-position of the 6-μm-wide regions. (*a*) Distance fraction of tip-directed movements (type 1), (*b*) grain-directed movements (type 5), and (*c*) nondirected movement (type 9). The three $*$ indicate the distance fraction for the sample of free organelles (plotted for practical reasons at $x = -0.5$ μm).

spectively $\overline{\langle \mathrm{Vel}_{\mathrm{type\ 1}} \rangle}$ and $\overline{\langle \mathrm{Vel}_{\mathrm{type\ 5}} \rangle}$, ranged from 0.30 μm/s to 1.06 μm/s. The values were clearly higher in the tube zone than in the tip zone (Table 1). Furthermore, the average mean velocities were ~20% higher for the grain-directed (type 5) than for the tip-directed movements (type 1) in all 14 6-μm-wide regions in the first 40 μm of the three cells.

Fig. 7 provides an overview of the average mean velocities for type 1 and 5 calculated for lanes with either uni or bidirectional movements, according to Fig. 6. In general, the velocities were lower in the bidirectional lanes than in the unidirectional lanes in the three cells (for significance see Table 2). Exceptions were found in a few 6-μm-wide regions with tip-directed movement in cell 1 (Fig. 7 *a*) and cell 2 (Fig. 7 *c*), or grain-directed movement in cell 2 (Fig. 7 *d*). Wilcoxon tests revealed that most differences in average mean velocity between the uni and bidirectional lanes were significant in the tip versus the tube region (Table 1) or among single 6-μm-wide regions (Table 2). The velocities were always lower for cell 3 than for the other two cells.

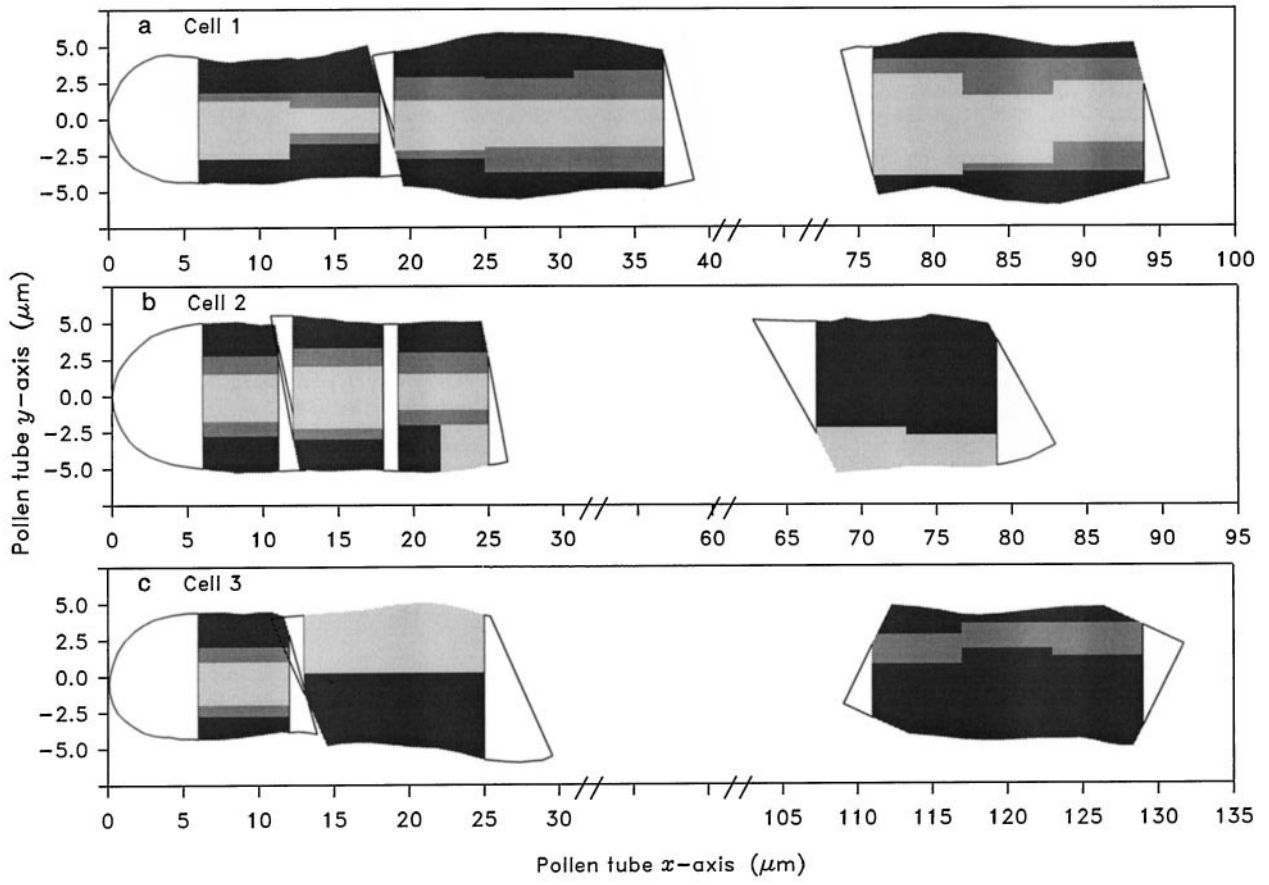

FIGURE 6 Representation of lanes showing movements with an axial component in three pollen tubes. The figure is based on arithmetical dissection. It reports on *x*-axial movements within 6-μm-wide regions, but does not comprise radial and undirected movements (types 3, 7, 9, and 10). The dark gray areas represent lanes with movements exclusively directed toward the tip (types 1, 2, and 8; unidirectional). The light gray areas represent lanes where movements exclusively with a component toward the grain are found (types 4–6; unidirectional). The middle gray lanes represent areas with bidirectional movements (types 1, 2, 4, 5, 6, and 8).

The overall pattern of absolute maximum velocities (no data shown) was similar to the one for average mean velocities. However, in a number of cases, the maximum values were not truly different for type 1 and type 5 movements (considering a margin in the measurements of ~0.12 μm/s; from de Win, 1997). For example, the highest velocity observed for type 5 movement was 2.13 μm/s (cell 1, region at 79 μm midpoint) and for type 1 it was 2.03 μm/s (same region).

These values for maximum velocity in tobacco measured over a large number of organelles were slightly lower than the corresponding value reported for individual organelles in pollen tubes of rye (2.58 μm/s), iris (2.47 μm/s), rosebay (4.0 μm/s) (Heslop-Harrison and Heslop-Harrison, 1987, 1988, 1990), and pine (velocity of the small subset of organelles showing transient linear movement: ~2.5 μm/s; calculated from data in de Win et al., 1996). The mean velocity is in the same order of magnitude for tobacco (this study) and lily (Iwanami, 1956): 0.3–0.6 μm/s in tobacco versus 0.6 μm/s in lily 20 μm from the tip, 0.7–1.1 μm/s for grain-directed movement at 70 μm from the tip in tobacco versus 0.7 μm/s in lily.

Cytoskeletal inhibitors

When cytochalasins were applied, the organelles ceased to move within a few seconds or minutes, depending on the concentration used. After 10 min of treatment, virtually all organelles appeared as fixed particles. Accordingly, quantitative analyses performed on 47 organelles treated with 2.0×10^{-5} M cytochalasin D showed a drastic inhibition of -directed- movement: the average mean velocity was 0.16 μm/s (SD = 0.05 μm/s), the Q_r was 1.7, and only 1 out of the 47 organelle trajectories had a *P* exceeding 0.9 (compare with control values in Fig. 3 *a*). Arithmetical dissection also revealed only one stretch of directed movement (in this case, type 1 movement). The inhibitory effect of cytochalasins was completely reversed by addition of fresh medium devoid of antagonist.

On the other side, when colchicine was added to the culture, even at an extremely high concentration (10^{-2} M), the cytoplasmic streaming remained unaffected also after 30 min of treatment, while growth proceeded.

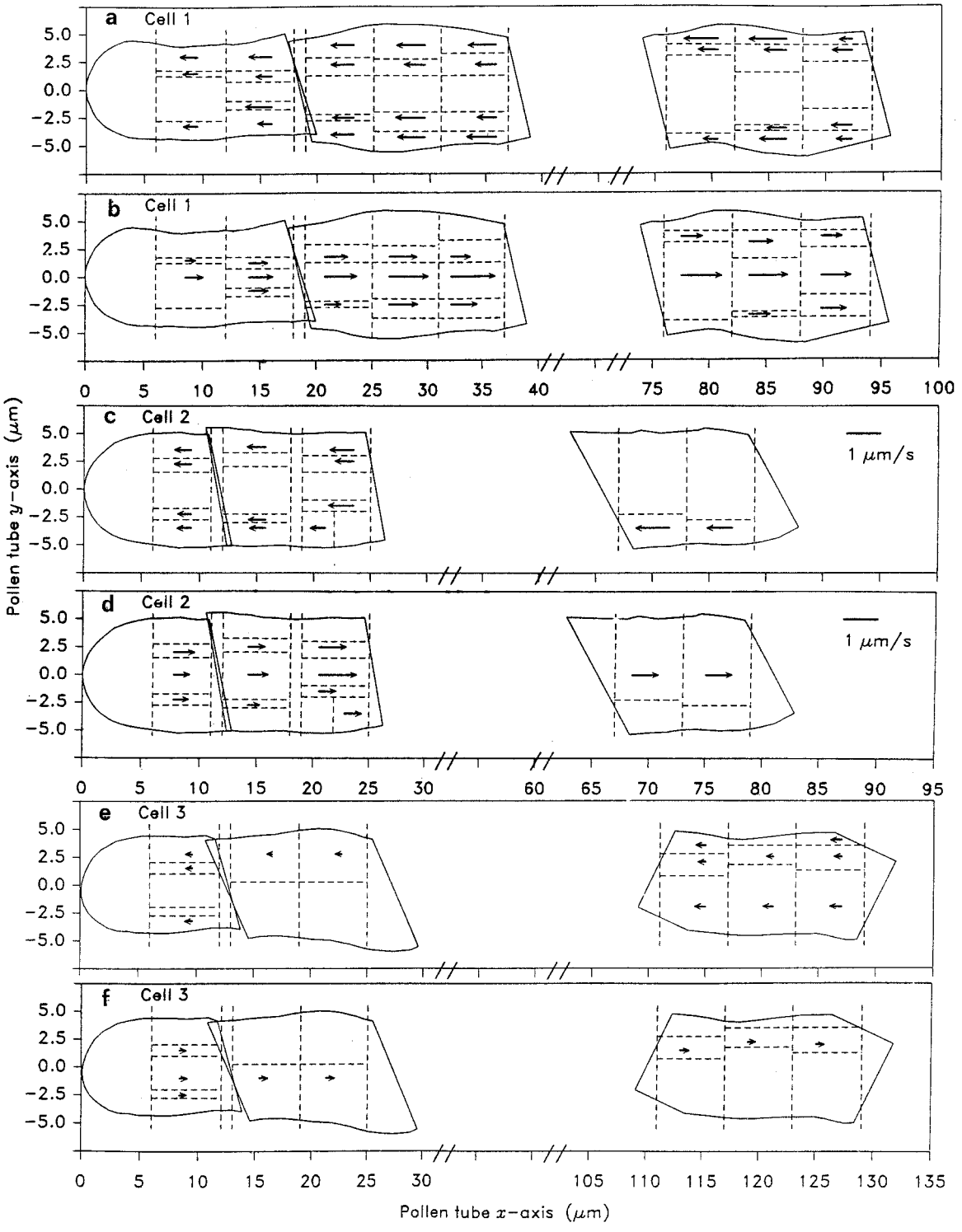

FIGURE 7 Average mean velocity in parts of trajectories with tip-directed (type 1; *a, c, e*) or grain-directed movements (type 5; *b, d, f*) within uni and bidirectional lanes of three pollen tubes. The contours of the cells are outlined by solid lines. The boundaries of the lanes, as defined in Fig. 6, are indicated by dashed lines. The values for mean average velocities are denoted by calibrated arrows.

DISCUSSION AND CONCLUSIONS

New methodology

Arithmetical dissection may be used to determine trends in motion vectors in both individual tracks and populations of tracks. This method is particularly appropriate to determine persistence in movement directionality when movement patterns are complex and irregular, and to evaluate relationships between movement and other parameters. This new method appears to be more powerful than the P or Q_r approach to

unravel organelle movements in pollen tubes. The method is easily adaptable to study two- or three-dimensional particle movement in other biological as well as nonbiological systems.

Analyses of reconstructed tracks

Detailed analysis of the reconstructed tracks confirmed the presence of an overall reverse fountainlike pattern of organelle movement in tobacco pollen tubes, compatible with the original descriptions given by Iwanami (1956) for lily, but with the difference that the individuality of movement could be established in a much more convincing way than previously expressed (Heslop-Harrison and Heslop-Harrison, 1988; Pierson et al., 1990; Emons et al., 1991). Each of the 900 reconstructed organelle tracks in tobacco pollen tubes had a unique course. The tracks showed great irregularities in shape and velocity, as established by calculating the grade of individuality of series of displacements and the occurrence of sudden accelerations and decelerations within a track. Although we do not have comparable data taken in a flow system, these results strongly indicate that bulk flow can only contribute marginally, if at all, to drive organelle movement in pollen tubes. Obviously, active processes play an important role in the motion of organelles in pollen tubes. Present data on inhibitors of the cytoskeleton are in full agreement with the earlier reports, which show that the movement of organelles is exclusively generated by the actomyosin system in pollen tubes of various species (reviewed in Steer and Steer, 1989; Pierson and Cresti, 1992). Compared to the movement of organelles along thick arrays of actin filaments in Characean giant algae (Williamson, 1993), vectorial movements of organelles in pollen tubes are irregular, sometimes even saltatory (see also de Win et al., 1997). Bundles of actin filaments may be discontinuous and slightly mobile in pollen tubes, as observed in a preliminary study in which living tobacco pollen tubes were labeled for filamentous actin (E. S. Pierson and F. Doris, unpublished data). Another source of variability in the movement patterns may be due to the fact that different classes of myosin may account for the translocation of different types of organelles (Miller et al., 1995). In addition, transient effects, such as steric hindrance, friction, and attachment to other particles, are expected to occur in the extremely dense cytoplasm of pollen tubes and to contribute to the observed unpredictable variations in the motility patterns.

Differentiation in movement patterns in pollen tubes

Analyses with the Q_r, the P, and the distance fractions in organelle populations of control pollen tubes show with significance that movements of organelles are randomlike in the tip and become gradually more vectorial and rapid with increasing distance from the tip. Since the same organelles, which most likely retain their proper myosin coating, circulate through the various regions of the tube, the differentiation in behavior is obviously spatially defined. The tip-to-base profile in movement types and organelle velocity probably reflects variations in density and length of bundles of actin filaments, and conform with the presence of a tip-localized high calcium domain in growing pollen tubes (Pierson et al., 1994, 1996; Malhó and Trewavas, 1996; Messerli and Robinson, 1997; Holdaway-Clarke et al., 1997), which is assumed to inhibit polymerization of globular actin into filaments. This assumption is in agreement with the observations made by Miller and co-workers (1996) on living and cryofixed lily pollen tubes. The behavior of organelles may also be influenced by local variations in cytosolic pH, as reported recently in the apex of lily pollen tubes (Feijó et al., 1998), and perhaps by preferential domains for certain actin isoforms too (McLean et al., 1990). Additional subcellular variations in physiological conditions are expected to influence the movement pattern of organelles, comparable to what has been described in other tip-growing cells, i.e., axons (Koles et al., 1982; Gulden et al., 1988).

The arithmetical dissection analysis unambiguously reveals a radial differentiation characterized by antigrade movement along the periphery of the pollen tube, retrograde in the center of the cell, and an intercalated zone with bidirectional movement. Since it is well-accepted that the shape and polarity of the actin filaments dictate the direction of organelle movement in plant cells (Emons et al., 1991; Williamson, 1993), and since myosins move to the +-end of the actin filaments, two predictions can be made on account of the results. First, actin filaments must have an axial arrangement along the long axis of the pollen tube, which indeed is in agreement with published microscopical observations (for a review see Pierson and Cresti, 1992; recent work on tobacco: Doris and Steer, 1996). Second, actin filaments must have an opposite polarity in the peripheral (according to the logics and theory: +-ends of actin filaments oriented toward the tip) than in the central cytoplasm (+-ends of actin filaments toward the grain). We propose that actin filament polarity is determined by a gradient of messages extending from the edge of the cell apex to the center of the tube. Polymerization of actin filaments—in a polar manner—is expected to take place at the site where the most apical lanes with directional movements are observed; that is, at ~6 μm from the growing tip in tobacco (Fig. 6), which corresponds to an area formed ~4 min earlier for a pollen tube growing 1.5 μm/min. The putative polymerization site of actin filaments coincides with the region boarding the vesiculate tip zone (compare with Heslop-Harrison and Heslop-Harrison, 1990, 1994; Derksen et al., 1995 a, b; Miller et al., 1996).

We observed that grain-directed movements were more rapid than tip-directed movements in the apex of the three cells (Fig. 7). Because pollen tubes may be regarded as a closed volume with respect to the organelles studied presently (only the exocytotic particles, the Golgi vesicles, were excluded from the sampling), the total number of measured organelles moving in antigrade direction must be equal to

the number of organelles moving in retrograde direction at a certain moment and certain distance from the tip. As a consequence, at steady state, the average velocity and total amount of organelles in a transversal section of the retrograde lanes must be in balance with the same parameters in the antigrade lanes. These deductions are in accord with variations in ultrastructural organization observed between candidate "fast" and "slow" lanes in lily pollen tubes (Lancelle and Hepler, 1992).

Arithmetical dissection indicates that the velocity is mostly slower and movement is more frequently interrupted in bidirectional lanes than in unidirectional ones. From data on *time fractions* reported in de Win (1997), we also know that nondirected movement occurs more often in bidirectional lanes. Therefore, it is likely that steric hindrance and shearing forces play a role between strings of organelles moving in opposite directions. Transient attachment between organelles and tubules of endoplasmic reticulum or other organelles have been repeatedly observed in living onion epidermis cells (Lichtscheidl and Url, 1987; Lichtscheidl and Weiss, 1988; Lichtscheidl, 1995), but also in the peripheral cytoplasm bordering large vacuoles in old lily pollen tubes (Pierson et al., 1990). In tobacco pollen tubes, physical association of organelles to other organelles or microtubules has been shown by electron microscopy (Derksen et al., 1995a). By contrast, slip-stream effects, or the absence of obstacles in the track of fast-moving organelles, may facilitate unrestricted, and therefore faster, movement in lanes with unidirectional movement.

Behavior of organelles after cytochalasin treatment or extrusion from the cell

Free organelles and organelles from cytochalasin-treated cells both have a low Q_r value and few trajectories with a high P value. These data coincide with the disruption or the inhibition of the actin cytoskeleton. The relatively rapid, though not focused, movements displayed by free organelles are in agreement with the microscopic observations that physical boundaries, which cause steric hindrance, are less prominent in extruded cytoplasm than in the cell. However, some constraints visibly persist. Their presence may clarify the Q_r value of 1.82, below the limit of 2.0 for ideal Brownian movements (for further theoretical discussion see Qian et al., 1991). It is reasonable to find hardly any directed movement in the sample of organelles from the cytochalasin-treated cell, as these particles are probably rigidly attached to the actin filaments. Altogether, the behavior of the organelles after cytochalasin treatment or extrusion from the cell emphasizes that an intact actin skeleton is essential to perform coordinated organelle movement. It is fascinating to realize the efficiency with which this extraordinary well-balanced motility system is implemented in pollen tubes.

Prof. Dr. W. Url and Dr. I. K. Lichtscheidl (University of Vienna) are kindly acknowledged for the use of superb microscopic video equipment and for stimulating discussion. The authors thank Th. de Boo (Medical Statistical Department, KUN) for giving statistical advice and H. van der Linden (Audiovisual Service, KUN) for the use of video recorders. Dr. D. D. Miller is kindly thanked for her scientific comments and suggestions to improve the English text.

The Netherlands Organization for Scientific Research provided support to AHNdW, and the University of Vienna to ESP to visit the Department of Plant Cell Physiology of the University of Vienna. Anonymous referees provided valuable criticisms that were followed to improve the manuscript.

REFERENCES

Cai, G., A. Moscatelli, C. Del Casino, and M. Cresti. 1996. Cytoplasmic motors and pollen tube growth. *Sex. Plant Reprod.* 9:59–64.

Chang, R. 1981. Diffusion. *In* Physical Chemistry with Applications to Biological Systems. MacMillan Publishing Company, New York. 83–89.

Derksen, J., T. Rutten, I. K. Lichtscheidl, A. H. N. de Win, E. S. Pierson, and G. Rongen. 1995a. Quantitative analysis of the distribution of organelles in tobacco pollen tubes: implications for exocytosis and endocytosis. *Protoplasma*. 188:267–276.

Derksen, J., T. Rutten, T., A. van Amstel, A. H. N. de Win, F. Doris, and M. W. Steer. 1995b. Regulation of pollen tube growth. *Acta Bot. Neerl*. 44:93–119.

de Win, A. H. N. 1997. Quantitative analysis of organelle movements in pollen tubes. Ph.D. thesis. Catholic University of Nijmegen. 146 pp.

de Win, A. H. N., B. Knuiman, E. S. Pierson, H. Geurts, H. M. P. Kengen, and J. Derksen. 1996. Development and cellular organization of *Pinus sylvestris* pollen tubes. *Sex. Plant Reprod.* 9:93–101.

de Win, A. H. N., E. S. Pierson, C. Timmer, I. K. Lichtscheidl, and J. Derksen. 1998. An interactive computer-assisted position acquisition procedure designed for the analysis of organelle movement in pollen tubes. *Cytometry*. 32:263–267.

de Win, A. H. N., M. Worring, J. Derksen, and E. S. Pierson. 1997. Classification of organelle trajectories using region-based curve analysis. *Cytometry*. 29:136–146.

Doris, F. P., and M. W. Steer. 1996. Effects of fixatives and permeabilisation buffers on pollen tubes: implications for localisation of actin microfilaments using phalloidin staining. *Protoplasma*. 195:25–36.

Emons, A. M. C., E. S. Pierson, and J. Derksen. 1991. Cytoskeleton and intracellular movement in plant cells. *In* Biotechnology, Current Progress. P. N. Cheremisinoff and L. M. Ferrante, editors. Technomic Publishing Company, Lancaster. 311–335.

Feijó, J. A., G. Hackett, J. Kunkel, and P. Hepler. 1998. Calcium and proton extracellular fluxes, growth oscillations and cytosolic pH in growing pollen tubes of *Lilium*. *Proceedings of the XVth International Congress on Sex and Plant Reproduction*. August 16–21. Wageningen. 26.

Gulden, J., D. G. Weiss, and B. Clasen. 1988. The velocity fluctuations of organelles transported in crustacean and human axons are random. *Eur. J. Cell Biol*. 46:24.

Heslop-Harrison, J., and Y. Heslop-Harrison. 1987. An analysis of gamete and organelle movement in the pollen tube of *Secale cereale* L. *Plant Sci*. 51:203–213.

Heslop-Harrison, J., and Y. Heslop-Harrison. 1988. Organelle movement and fibrillar elements of the cytoskeleton in the angiosperm pollen tube. *Sex. Plant Reprod*. 1:16–24.

Heslop-Harrison, J., and Y. Heslop-Harrison. 1990. Dynamic aspects of apical zonation in the angiosperm pollen tube. *Sex. Plant Reprod*. 3:187–194.

Heslop-Harrison, J., and Y. Heslop-Harrison. 1994. Restoration of movement and apical growth in the angiosperm pollen tube following cytochalasin-induced paralysis. *Phil. Trans. R. Soc. Lond. B*. 331:225–235.

Holdaway-Clarke, T. L., J. A. Feijó, G. R. Hackett, J. G. Kunkel, and P. K. Hepler. 1997. Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *Plant Cell*. 9:1999–2010.

Iwanami, Y. 1956. Protoplasmic movement in pollen grains and tubes. *Phytomorphology*. 6:288–295.

Iwanami, Y. 1959. Physiological studies on pollen. *Yokohama Municipal University (C-34, Biol. 13)*. 116:1–137.

Jarosch, R. 1956. Die Impulsrichtungsänderungen bei der Induktion der Protoplasmaströmung. *Protoplasma*. 67:478–486.

Kohno, T., S. Chaen, and T. Shimmen. 1990. Characterization of the translocator associated with pollen tube organelles. *Protoplasma*. 154: 179–183.

Kohno, T., and T. Shimmen. 1987. Ca^{2+}-induced fragmentation of actin filaments in pollen tubes. *Protoplasma.* 141:177–179.

Koles, K. J., K. D. McLeod, and R. S. Smith. 1982. The determination of the instantaneous velocity of axonally transported organelles from filmed records of their motion. *Can. J. Physiol. Pharmacol*. 60: 670–679.

Lancelle, S. A., and P. K. Hepler. 1988. Cytochalasin-induced ultrastructural alterations in *Nicotiana* pollen tubes. *Protoplasma*. (Suppl.) 2:65–75.

Lancelle, S. A., and P. K. Hepler. 1992. Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum. Protoplasma*. 167:215–230.

Lichtscheidl, I. K. 1995. Organization and dynamics of plant cell cytoplasm; inner epidermal cells *Allium cepa* bulb scales investigated by modern microscopical techniques. *Wissenschaftlicher Film (Wien)*. 47: 1–151 (book and films).

Lichtscheidl, I. K., and W. G. Url. 1987. Investigation of the protoplast of *Allium cepa* inner epidermal cells using ultraviolet microscopy. *Eur. J. Cell Biol.* 43:93–97.

Lichtscheidl, I. K., and D. G. Weiss. 1988. Visualization of submicroscopic structures in the cytoplasm of *Allium cepa* inner epidermal cells by video-enhanced contrast light microscopy. *Eur. J. Cell Biol.* 46: 376–382.

Mace, A. E. 1964. Sample-Size Determination. Reinhold Publishing Corporation, New York.

Malhó, R., N. D. Read, M. S. Pais, and A. J. Trewavas. 1994. Role of cytosolic free calcium in reorientation of pollen tube growth. *Plant J.* 5:331–341.

Malhó, R., and A. J. Trewavas. 1996. Localized apical increases of cytosolic free calcium control pollen tube orientation. *Plant Cell*. 8:1935–1949.

Mascarenhas, J. P. 1993. Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell.* 5:1303–1314.

McLean, B. G., S. Huang, E. C. McKinney, and R. B. Meagher. 1990. Plant contain highly divergent actin isovariants. *Cell Motil. Cytoskel*. 17: 276–290.

Messerli, M., and K. R. Robinson. 1997. Tip-localized Ca^{2+} pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum. J. Cell Sci.* 110:1269–1278.

Miller, D. D., S. A. Lancelle, and P. K. Hepler. 1996. Actin microfilaments do not form a dense meshwork in *Lilium longiflorum* pollen tube tips. *Protoplasma.* 195:123–132.

Miller, D. D., S. P. Scordilis, and P. K. Hepler. 1995. Identification and localization of three classes of myosins in pollen tubes of *Lilium longiflorum* and *Nicotiana alata. J. Cell Sci*. 108:2549–2563.

Overstreet, J. W., D. F. Katz, F. W. Hanson, and J. R. Fonseca. 1979. A simple inexpensive method for objective assessment of human sperm movement characteristics. *Fertil. Steril*. 31:162–172.

Pierson, E. S., and M. Cresti. 1992. Cytoskeleton and cytoplasmic organization of pollen and pollen tubes. *Int. Rev. Cytol*. 140:73–125.

Pierson, E. S., I. K. Lichtscheidl, and J. Derksen. 1990. Structure and behaviour of organelles in living pollen tubes of *Lilium longiflorum. J. Exptl. Bot*. 41:1461–1468.

Pierson, E. S., D. D. Miller, D. A. Callaham, A. M. Shipley, B. A. Rivers, M. Cresti, and P. K. Hepler. 1994. Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: effect of BAPTA-type buffers and hypertonic media. *Plant Cell*. 6:1815–1828.

Pierson, E. S., D. D. Miller, D. A. Callaham, J. van Aken, G. Hackett, and P. K. Hepler. 1996. Tip-localized calcium entry fluctuates during pollen tube growth. *Dev. Biol*. 174:160–173.

Qian, H., M. P. Sheetz, and E. L. Elson. 1991. Single particle tracking: analysis of diffusion and flow in two-dimensional systems. *Biophys. J*. 60:910–921.

Steer, M. W., and J. M. Steer. 1989. Pollen tube tip growth. *New Phytol*. 111:323–358.

Taylor, L. P., and P. K. Hepler. 1997. Pollen germination and tube growth. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:461–491.

Williamson, R. E. 1993. Organelle movements. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 44:181–202.

Yokota, E., A. R. McDonald, B. Liu, T. Shimmen, and B. A. Palevitz. 1995. Localization of a 170 kDa myosin heavy chain in plant cells. *Protoplasma*. 185:178–187.