Exclusion of a Proton ATPase from the Apical Membrane Is Associated with Cell Polarity and Tip Growth in Nicotiana tabacum Pollen Tubes

Ana C. Certal, a Ricardo B. Almeida, a, b Lara M. Carvalho, a Eric Wong, c Nuno Moreno, d Erwan Michard, a Jorge Carneiro, d Joaquin Rodriguez-Leon, a, d Hen-Ming Wu, c Alice Y. Cheung, c and Jose A. Feijoo a, b, 1

a Instituto Gulbenkian de Ciencia, Centro de Biologia do Desenvolvimento, 2780-156 Oeiras, Portugal
b Departamento Biologia Vegetal, Faculdade de Ciencias, Universidade de Lisboa, Campo Grande, 1700 Lisboa, Portugal
c Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts, 01003
d Centre de Medicina Regenerativa de Barcelona, E-08003 Barcelona, Spain

Polarized growth in pollen tubes results from exocytosis at the tip and is associated with conspicuous polarization of Ca2+, H+, K+, and Cl−-fluxes. Here, we show that cell polarity in Nicotiana tabacum pollen is associated with the exclusion of a novel pollen-specific H+-ATPase, Nt AHA, from the growing apex. Nt AHA colocalizes with extracellular H+ effluxes, which revert to influxes where Nt AHA is absent. Fluorescence recovery after photobleaching analysis showed that Nt AHA moves toward the apex of growing pollen tubes, suggesting that the major mechanism of insertion is not through apical exocytosis. Nt AHA mRNA is also excluded from the tip, suggesting a mechanism of polarization acting at the level of translation. Localized applications of the cation ionophore gramicidin A had no effect where Nt AHA was present but acidified the cytosol and induced reorientation of the pollen tube where Nt AHA was absent. Transgenic pollen overexpressing Nt AHA-GFP developed abnormal callose plugs accompanied by abnormal H+ flux profiles. Furthermore, there is no net flux of H+ in defined patches of membrane where callose plugs are to be formed. Taken together, our results suggest that proton dynamics may underlie basic mechanisms of polarity and spatial regulation in growing pollen tubes.

INTRODUCTION

Asymmetry and polarization are crucial not only for development but also for almost every functional aspect of cells. Various mechanisms have evolved to establish and maintain cellular polarity, which, in most cases, are based on asymmetrical distribution of specific proteins or on higher-order organization of cellular structures. Recently, the Par (Partition-defective) family of proteins has frequently been assigned an essential role in mammalian systems, notably in axon polarization (Benton and Johnston, 2003; Shi et al., 2003), but other protein families have also been implicated, namely, proteins in the PI3K-associated pathway (Horiguchi et al., 2006) and small GTPases (Wells et al., 2006). Despite increasing insight into the regulatory interactions of these proteins, some fundamental aspects still need to be resolved as, in most reports, the mechanisms through which polarization is achieved and maintained are not established.

Pollen tubes are cells that display an extreme example of cellular polarity and growth control, growing exclusively at the tip by means of apical exocytosis. They maintain impressive growth rates of up to 4 μm/s for several millimeters or centimeters, depending on the species, without ever dividing and are one of the fastest elongating cells in nature. Despite being a bona fide plant cell with an external polysaccharide wall, several parallels with neurite outgrowth have been established (Palanivelu and Preuss, 2000; Bicker, 2005); consequently, they have been used as a model to analyze various aspects of cell polarization (reviewed in Feijo et al., 2004; Boavida et al., 2005a, 2005b). Like most mammalian polarized cells, pollen tubes were shown to have an active small GTPase-based mechanism of actin cytoskeleton regulation (Fu et al., 2001; Chen et al., 2003; Gu et al., 2005; Hwang et al., 2005), but most remarkably and to some extent uniquely, they have been shown to have a conspicuous polarization of ion fluxes and gradients, presumably established by specific transporters (reviewed in Holdaway-Clarke and Hepler, 2003; Feijoo et al., 2004). In this regard, pollen has successfully been used as a cellular model to study ion dynamics and electric currents as key components in the control of growth and morphogenesis (Weisenseel et al., 1975; Malho et al., 1992a; Feijoo et al., 1995, 1999; Holdaway-Clarke et al., 1997; Holdaway-Clarke and Hepler, 2003). As early as 1975, Weisenseel et al. identified the germinated pollen grain as an electric dipole with the grain functioning as a source of a cationic current, that is, cations leave the grain and enter at the tip. It has recently been established that at least Ca2+, H+, K+, and Cl− are part of this current and that their extracellular fluxes are conspicuously polarized, presumably by polarization of their underlying transporters (Holdaway-Clarke et al., 1997; Feijoo et al., 1999; Messerli et al., 1999; Zonia et al., 2001, 2002). Internal apically polarized

1 Address correspondence to jfeijo@fc.ul.pt.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jose A. Feijoo (jfeijo@fc.ul.pt).

Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.106.047423
gradients of \( \text{Ca}^{2+} \) and \( \text{H}^+ \) have been correlated with the growth properties of these cells (Feijó et al., 1995, 1999; Holdaway-Clarke et al., 1997; Messerli et al., 1999; reviews in Feijó et al., 2004; Boavida et al., 2005b). In all these studies, these ions were correlated with some level of fundamental signaling mechanism, namely, through the Rop/Rac GTPase and the IP$_3$ and IP$_4$ pathways. Here, we will focus on the role of protons (\( \text{H}^+ \)).

By imaging with fluorescent probes, lily (\textit{Lilium longiflorum}) pollen tubes were shown to have a growth-dependent acidic tip and, immediately adjacent to the growing apex, a constitutive alkaline band that spatially correlates with the clear zone, a cytoplasmic domain devoid of large organelles. Furthermore, extracellular \( \text{H}^+ \) fluxes were found to correlate well with this pattern, with strong influxes in the apex, and stronger effluxes around the alkaline band (Feijó et al., 1999). More recently, detailed studies of actin cytoskeleton dynamics have suggested that this structural correlation is the basis for actin remodeling and polymerization (Chen et al., 2002; Cárdenas et al., 2005; Lový-Wheeler et al., 2006).

Protons have long been hypothesized as being a possible integrator of intracellular pathways (Felle, 2001). Cells live in aqueous media. Because water spontaneously ionizes, cells are surrounded by protons and any change in the \( \text{H}^+ \) aqueous media. Because water spontaneously ionizes, cells are integrator of intracellular pathways (Felle, 2001). Cells live in aqueous media. Because water spontaneously ionizes, cells are surrounded by protons and any change in the \( \text{H}^+ \) concentration (pH) will have an impact on a variety of molecules in different ways. Being both a substrate and a product in numerous metabolic reactions, protons fulfill a crucial regulatory role in the cell. Therefore, from a fine regulation standpoint, \( \text{H}^+ \) homeostasis is probably the most basic and effective common denominator between all living cells. Numerous signaling roles have been assigned to \( \text{H}^+ \) in plant physiology (Felle, 2001). The actin cytoskeleton has been thought to be affected by pH through specific actin binding proteins like ADF (Condeelis, 2001; Chen et al., 2002). The molecular description of the \( \text{H}^+ \) channel has also shed light on some of its fundamental properties for cell physiology (Ramsey et al., 2006; Sasaki et al., 2006). It is conceivable that localized/polarized insertion of specific \( \text{H}^+ \) transporters could be a fundamental link between diverse cellular polarization systems by affecting basic mechanisms in a spatial and temporarily synchronized way. In a previous report (Feijó et al., 1999), we proposed a theoretical model to explain the observed proton patterns in pollen tubes based on the exclusion of \( \text{H}^+ \)-ATPases from the apical domain of the pollen tube membrane. This model was later generalized to other types of cells as the possible molecular basis underlying apical cell growth (Palmgren, 2001; Feijó et al., 2004).

In this report, using tobacco (\textit{Nicotiana tabacum}) as a model species, we cloned a pollen plasma membrane \( \text{H}^+ \)-ATPase (Nt AHA). By green fluorescent protein (GFP) labeling, this protein was shown to be absent from the apical membrane, in a pattern that closely correlated with the \( \text{H}^+ \) extracellular fluxes, suggesting that Nt AHA is responsible for the extracellular fluxes. We aimed to mechanistically couple the molecular results with available cell physiology data. The prediction would be that the expression of Nt AHA would be temporally and spatially coupled with macroscopic characteristics of pollen tube growth; therefore, if the systems’ \( \text{H}^+ \) dynamics were perturbed, they would produce consistent responses in measurable growth-related parameters. We tested this by a combination of advanced imaging, fluorescence recovery after photobleaching (FRAP), cytokinematics, electrophysiology, and local ionophore challenge. Our results are consistent with the hypothesis that \( \text{H}^+ \) modulated cellular mechanisms involving a tight cellular control of \( \text{H}^+ \) dynamics are an essential part of the pollen tube growth mechanism that establishes and maintains polarity in these cells.

**RESULTS**

**Extracellular H\(^+\)** Fluxes in Tobacco Pollen Tubes Are Mediated by Plasma Membrane P-Type H\(^+\) Pumps

Because the predicted model for \( \text{H}^+ \) flux pattern in pollen tubes was based on the selective exclusion of \( \text{H}^+-\text{ATPase} \) from the tip (Feijó et al., 1999), we decided to test this hypothesis by investigating the distribution of these pumps in tobacco pollen tubes. We first performed a pharmacological screen, testing the effect of inhibitors for the main types of membrane pumping \( \text{H}^+ \)-ATPases on extracellular \( \text{H}^+ \) fluxes. Population effects for the four major proton pump inhibitors were assessed in pollen by calculating the concentration required for 50% inhibition (IC$_{50}$) of pollen tube growth. The inhibitors used and their respective IC$_{50}$ values are set forth in Supplemental Table 1 online. To evaluate the effect of these inhibitors on extracellular \( \text{H}^+ \) fluxes in actively growing pollen tubes, we measured the fluxes before and after adding the drug. The percentage of inhibition obtained for the different inhibitors varied significantly depending on the specific experiment. The most relevant \( \text{H}^+ \) flux activity was found to be orthovanadate dependent (see Supplemental Figure 1 online).

Sodium orthovanadate produced the highest inhibition of \( \text{H}^+ \) fluxes (by 60 to 80%), especially at the flanks adjacent to the apex (80%) (see Supplemental Figure 1 online), suggesting that plasma membrane P-type \( \text{H}^+ \)-ATPases are involved in the \( \text{H}^+ \) flux pattern observed. Although at a lower level, all other inhibitors also produced some flux inhibition that, however, was not as pronounced as for orthovanadate and varied much between experiments. These data established P-type \( \text{H}^+ \)-ATPases as strong candidates for pumps underlying \( \text{H}^+ \) fluxes in tobacco pollen tubes.

**Nt AHA Encodes a Plasma Membrane H\(^+\) Pump in Tobacco Pollen Tubes**

We isolated and cloned Nt AHA (for Autoinhibited \( \text{H}^+ \)-ATPase) (Baxter et al., 2003), an \( \text{H}^+ \)-ATPase from an \textit{N. tabacum} pollen cDNA library. The cDNA sequence was 3347 bp, and it encoded a deduced amino acid sequence of 951 residues.

Highly conserved domains in P-type \( \text{H}^+ \)-ATPases have been identified in Prosites databases (Falquet et al., 2002): one N-terminal cation transporter/ATPase domain, one E1-E2 ATPase domain, and one hydrolyase domain. Membrane protein structure prediction software (TopPredII; Claros and von Heijne, 1994) revealed a putative topology of 10 transmembrane domains, very similar to the one obtained for \textit{Arabidopsis thaliana} AHA1 using the same method (see Supplemental Figure 2 online). AHA nucleotide and predicted amino acid sequences were compared with sequences in the GenBank database, and strong similarity...
was found only with H\(^{+}\)-ATPases from other species, which lead us to assume that Nt AHA is a bona fide H\(^{+}\)-ATPase.

Two plant species in which a large number of H\(^{+}\)-ATPases are known and characterized are Arabidopsis (AHAs) and Nicotiana plumbaginifolia (plasma membrane ATPases [PMAs]). Total amino acid sequence alignment of AHA with both Arabidopsis and N. plumbaginifolia H\(^{+}\)-ATPases clusters Nt AHA with AHA9, AHA6, AHA8, PMA5, and PMA6 (see Supplemental Figure 3 and Supplemental Data Set 1 online), thus determining that, according to Palmgren (2001), Nt AHA belongs to Subfamily III. Transcriptome data from Arabidopsis pollen (Pina et al., 2005) revealed that all AHA isoforms that cluster with Nt AHA are highly expressed in pollen (AHA8 showing the highest expression level of all pollen-expressed isoforms) and that two isoforms, AHA6 and AHA9, are pollen specific. PMA6 expression was also observed in pollen grains and tubes (Oufattole et al., 2000), and PMA5 was shown to be expressed in pollen (Lefebvre et al., 2005). This suggests that P-type H\(^{+}\)-ATPases may have evolved toward tissue expression/specificity.

**Nt AHA-GFP Accumulates in the Plasma Membrane of the Pollen Tube but Is Excluded from the Apex**

To verify intracellular distribution, Nt AHA cDNA was subcloned into an expression vector downstream of the pollen-predominant LAT52 promoter (Twell et al., 1990) and upstream of a pH-insensitive enhanced version of GFP (Chiu et al., 1996). The resulting construct was used to transform pollen grains by particle bombardment, and transient expression of Nt AHA-GFP was assessed by two-photon microscopy in growing pollen tubes after 3 to 4 h of germination. In parallel, transgenic lines stably overexpressing the fusion construct (LAT52:NtAHA-GFP) were also raised.

Both transient and stable expression of Nt AHA-GFP revealed that GFP labeling localized to the plasma membrane (Figure 1B), as would be expected for a bona fide proton pump. This labeling pattern was clearly distinct from the control situation with GFP alone (Figure 1A), which shows a diffuse, homogeneous labeling in the cytosol. To eliminate the possibility that the signal is located in the cell wall, we plasmolyzed transformed pollen tubes with 30% sucrose. This confirmed that labeling was restricted to the plasma membrane of both the pollen grain (Figure 1C) and tube (Figure 1D).

Analysis of the growing tip of transformed pollen tubes showed a consistent decrease of the membrane labeling close to the apex, roughly coincident with the apical clear zone. In all pollen tubes, GFP labeling was absent from the membrane at the extreme apex (Figures 1E and 1F). Less conspicuously, a highly dynamic V-shape intracellular diffuse labeling could be observed in growing tubes (Figure 1E; see Supplemental Movie 1 online). The shape, size, and location of this intracellular fluorescence are coincident with the V-shaped cone of vesicles present in the apex of growing pollen tubes (e.g., Derksen et al., 1995; Parrot et al., 2001, 2003; de Graaf et al., 2005). This apical accumulation of Nt AHA-GFP is lost in nongrowing pollen tubes, which nevertheless maintain a similar membrane localization pattern of Nt AHA-GFP as elongating tubes (Figure 1F). To exclude the possibility of this intracellular labeling being an artifact of the over-expression of an active pump, we transiently expressed a catalytically dead mutant of Nt AHA-GFP in pollen tubes, where the essential Asp in the core sequence DKTGT was substituted with Asn. As expected, this inactive version of the tagged pump showed the same localization as the normal pump (see Supplemental Figure 4 online), indicating that the intracellular localization is not activity dependent.

**Nt AHA Distribution Correlates with H\(^{+}\) Efflux Activity, and Its Fluorescence Intensity Is Periodically Distributed**

Sequential transversal planes through the tip of a transgenic pollen tube show the loss of membrane fluorescence close to the apex (Figure 2A, plane 13). Quantification of this labeling by pixel intensity line scans along the membrane enabled us to determine the decrease of labeling toward the tip and the absence of labeling at the apex (Figure 2B).

In scans performed at the tip region, a trend was always present in both sides of the tube toward the tip (i.e., the labeling showed a gradual decrease toward the apex). Figure 2B shows this characteristic pattern with the decrease of labeling starting...
Figure 2. Fluorescence Profiling of Nt AHA-GFP Labeling.

(A) Sequential transversal planes (1 μm apart) of the tip of a growing Nt AHA-GFP transgenic pollen tube; plasma membrane Nt AHA-GFP labeling decreases toward the apex (plane 13) where only intracellular labeling is detected.

(B) Membrane labeling profile with fluorescence decay at ~15 μm from the tip (arrows) at both sides of a pollen tube and almost no labeling detected at the very tip. a.u., arbitrary fluorescence intensity units.

(C) Spectral analysis of a labeling profile where a fluctuation pattern in the fluorescence was observed; the bottom graph shows a significant frequency peak corresponding to a spatial period of ~10 μm.

(D) H⁺ flux profile of a LAT52:NtAHA-GFP tube showing that the spatial distribution of fluxes matches the fluorescence profile for these proton pumps.
at ∼20 µm from the tip on the left side and at ∼15 µm from the tip on the right side (arrows). In this particular case, the tube was turning left, which may account for the difference in these distances. At ∼5 µm from the tip, another sudden drop in fluorescence is clearly observed.

We further quantified this drop of fluorescence by randomly selecting 20 pollen tubes and normalizing them to an 8-bit referential (0 to 255 arbitrary fluorescence intensity units) in a way that gain and black level were regulated so that <1% of the pixels have either 0 or 255 values. Transversal line scans were then generated at 10 and 60 µm back from the tip. The 10-µm distance was chosen because the membrane pixels come out as defined peaks in the line scan at this point, allowing clear distinction from the cytosolic signal. Under these conditions, the cytosol presents a fluorescence of 50 ± 20 arbitrary units, a value not significantly different from the membrane at 10 µm (80 ± 18, analysis of variance, P > 0.05) but significantly different from the membrane at 60 µm (230 ± 12, P < 0.01).

To directly correlate this fluorescence profile with the H⁺ flux profile, extracellular H⁺ fluxes were measured in LAT52:NtAHA-GFP pollen tubes (Figure 2D). We could determine that at the same distances from the tip (10 µm and 15 to 20 µm), where major drops of fluorescence were detected, major changes in H⁺ fluxes are observed. At 10 µm from the tip, there is a decrease in the H⁺ influx compared with the tip, and at 15 to 20 µm, the influx is reverted to an efflux. This result suggests that Nt AHA activity may be responsible for the H⁺ effluxes generated by pollen tubes and confirmed the previous prediction that absence of effluxes at the tip may be due to selective exclusion of this pump from the apex.

Interestingly, in some fluorescence profiles, a fluctuation pattern was readily observed with a spatial periodicity of ∼10 to 20 µm. Spectral analysis was then used to statistically verify this putative spatial period. The analysis was performed on crude data, on smoothed data (box smooth = 26, i.e., each point represents the average of the neighboring 20 points), and on detrended data (original data subtracted from box-smoothed data). The periodogram showed that most tubes have periods from 10 to 40 µm (Figure 2C). This period is similar to the spatial equivalent of growth rate oscillations typical in tobacco pollen tubes (Moreno et al., 2007).

**Nt AHA-GFP Moves toward the Tip after Growth**

Exocytosis is believed to occur at the tip of pollen tubes by fusion of secretory vesicles with the plasma membrane and subsequent release of cell wall material (Derkson et al., 1995). Other molecules, such as membrane proteins, are also thought to be incorporated there (Schött et al., 2004). However, the Nt AHA-GFP expression profile revealed a membrane labeling excluded from the tip, suggesting that these proteins may be delivered to the membrane by a different route. We used FRAP to investigate the dynamics of Nt AHA-GFP in the membrane plane and thus to infer about possible insertion mechanisms.

On a first approach, we optimized the methods and radiation levels to accomplish the minimal perturbation possible to cells. Comparison between confocal and two-photon excitation showed the latter to be superior in terms of power availability to bleach but also remarkably in terms of the viable parameters quantified (streaming and growth rate). Given the restricted subtomoliter focal volume of two-photon excitation, this result is not surprising (Feijo and Moreno, 2004). Wavelength also proved an important factor, and 800 nm was determined as an optimal value. Even on these strict controlled and tested conditions, the growth rate was always somehow affected by the imaging. Again, this result is not unexpected given the enormous quantity of energy needed to bleach and the presumably massive formation of singlet oxygen species during the process but becomes specially visible in a rapid growing cell like a pollen tube. Streaming, on the other hand, recovered in a matter of seconds, which allows the conclusion that most FRAP experiments published in the literature will likely have the same sort of deleterious effects, but they remain unnoticed due to the lack of a sensitive enough vital parameter like cellular growth.

Under these irradiation-optimized conditions, FRAP was performed on the plasma membranes of transformed pollen tubes expressing Nt AHA-GFP at ∼50 µm from the tip (Figure 3A). Bleaching closer to the tip systematically led to pollen tube bursting, suggesting a higher susceptibility to photon excitation in this membrane domain. In tubes where FRAP was successful (n = 13), fluorescence was always shown to move longitudinally in the membrane plane toward the tip. Fluorescence movement during recovery was assessed using automated digital image analysis. A graphical depiction of this directional movement can be clearly observed in Figure 3B (see Supplemental Movie 2 online). Quantification led to an estimate of an average moving rate for a given fluorescent molecule of 0.33 ± 0.14 µm min⁻¹ (n = 8), similar to rates reported for other tethered, nonfreely moving, membrane proteins (Jesaitis and Yguerabide, 1986; Adams et al., 1998). After bleaching, pollen tubes recovered to a growth rate of 0.5 to 1.0 µm min⁻¹. In nongrowing but alive tubes (i.e., tubes showing cytoplasmic streaming), the bleached area recovered fluorescence but did not change position, which leads us to believe that the movement is not a passive consequence of dragging by the cytoplasmic streaming but a direct consequence of the growth process. These results seem to point to a putative correlation between the movement rate of these proteins in the membrane and the pollen tube growth process.

**Nt AHA Polarization Correlates with Nt AHA-mRNA Exclusion from the Tip Region**

To evaluate if polarization of Nt AHA is due to polarized translation, Nt AHA mRNA was detected in early germinated and fully grown pollen tubes (200 to 300 µm long) by whole-mount in situ hybridization. Three different riboprobes were used to target different regions in the Nt AHA sequence: Probe 1 was directed to a relatively variable region spanning from nucleotide 1209 to nucleotide 1643, Probe 2 included a more conserved region among known H⁺-ATPases, from nucleotide 574 to nucleotide 1486, and Probe 3 was designed to target the C-terminal region from nucleotide 2109 to nucleotide 3347. Sense probes were used as controls.

Standard nonradioactive whole-mount in situ hybridization protocols use digoxigenin (DIG)-labeled probes and anti-DIG antibodies conjugated with a tag to detect DIG. Since plant cells do not readily internalize large molecules like antibodies without
treatments that degrade the cell wall and there is only one report of a successful pollen whole-mount hybridization using DIG-labeled probes (Torres et al., 1995), we developed a protocol for whole-mount in situ hybridization in pollen in which the probes were directly labeled with UTP-AlexaFluor488 and the signal was then detected using two-photon microscopy. Using a high magnesium fragmentation buffer, UTP-AlexaFluor488–labeled probes were heat-sheared into a pool of 40- to 150-bp fragments before being applied to pollen tubes for hybridization.

Two of the antisense probes detected a similar pattern of Nt AHA mRNA (Figure 4A) clearly distinct from controls (Figures 4E and 4F), whereas antisense Probe 1 did not show any distinct pattern. Nt AHA mRNA labeling showed a spotty/thready pattern scattered along the whole length of the tube except for the tip region (Figure 4A). In tubes showing vacuoles up to the tip, the labeling was homogeneously distributed (Figure 4B). This spotty pattern of Nt AHA mRNA labeling is similar to what was described in other cell types (Bratu et al., 2003) and is clearly evident in zoomed images (Figure 4C). These spots could be visualized at a plane immediately below the plasma membrane (arrows in Figure 4D). As a membrane protein, Nt AHA translation and folding is expected to occur in the endoplasmic reticulum (ER). An ER pattern was previously reported in Nicotiana pollen tubes in which lamellar rough ER localizes everywhere but the tip, whereas smooth tubular ER elements are present up to the tip (Cresti et al., 1992; Lancelle and Hepler, 1992). This suggests that polarization of Nt AHA translation may occur due to the exclusion of rough ER from the tip and may contribute to the maintenance of the polarized protein profile of Nt AHA.

The Nt AHA–Depleted Apical Domain Is Susceptible to Proton Influx, Inducing Reorientation of the Growth Axis

We hypothesized that Nt AHA depletion from the apical membrane would create a tip membrane/cytoplasm domain with more susceptibility to local ionic fluxes and, thus, presumably, more sensitive to small imposed ion gradients. In practical terms, the absence of Nt AHA, and consequently the absence of its important H⁺ efflux (pumping) activity, is reflected by a net influx of H⁺ into the apex. We thus decided to test this sensitization hypothesis by inducing an H⁺ influx with gramicidin A, which is a highly hydrophobic peptide antibiotic that incorporates in lipid membranes as end-to-end-bound homodimers to form a highly permeable monovalent cation pore (Hille, 2001). Given the fast moving rate of protons, the permeability of gramicidin to H⁺ was shown to be one magnitude higher than that of potassium (K⁺), the only other ion in the germination medium with a presumable gradient that could induce its movement through the pore (the rate of permeability of H⁺ over K⁺ is 12.8; Urban et al., 1980). The predicted mechanism of the ionophore involves the location of two ions and eight to 10 molecules of water inside the pore (Hille, 2001). Combined with the strong pH gradient across the membrane (1.5 to 2.0 orders of magnitude), we expected that by applying gramicidin to growing pollen tubes we could induce a localized perturbation in the internal proton homeostasis through the entrance of additional protons into the cell in a specific membrane location.

Dose–response curves for gramicidin A versus pollen tube growth revealed an increased growth rate with gramicidin concentrations from 0.5 to 1.0 μM, showing toxicity above 10 μM (see Supplemental Figure 5 online).

The high hydrophobicity of the drug made it impossible to establish artificial gradients. We thus developed a protocol by which gramicidin A is directly applied to pollen tubes. The structure of the pollen cell wall in tobacco was shown by ultrapressure freezing scanning electron microscopy to correspond to a spongy, highly fenestrated, and thus permeable structure (Jan Derksen, personal communication). We thus assumed that direct application of the drug to the cell wall would be sufficient to form channels in the plasma membrane. In situ application was thus performed using glass micropipettes back-filled with different DMSO dilutions of gramicidin A.
The response of pollen tubes to gramicidin A was concentration dependent (Table 1). When applying drug concentrations above 65 μM at different points along the pollen tube, tubes immediately burst on contacting the drug (n = 4). With concentrations between 30 and 50 μM, tubes died either by bursting; 10 to 15 s after contact with the drug (n = 3; see Supplemental Movie 3 online) or with the entire cytoplasm being instantly pushed to the tip and frozen (n = 4; see Supplemental Movie 4 online). With concentrations below 20 μM, tubes showed no response to the drug. However, using a concentration of 25 μM, different responses were observed, depending on where the drug was administered. No response was observed (n = 6) when applying the drug at the tube shank (100 μm from the apex, where Nt AHA is present), but whenever this application was done laterally in the tip flanks (within 10 to 15 μm from the apex, where Nt AHA is absent), tubes turned at an angle of ~60° to 80° and grew toward the point of drug application (n = 5; see Supplemental Movies 5 and 6 online). All pollen tubes stopped growing ~3 min after the drug was applied and growth was never resumed. Attempts of secondary applications of gramicidin A to the opposite side resulted in immediate growth arrest or tube bursting. Controls using DMSO alone in the same lateral sites at the tip produced no response (see Supplemental Figure 6 online). Imaging of intracellular H⁺ concentration (Michard et al., 2008) further showed that the drug indeed provoked the entry of protons (Figure 5L). While normally growing tubes exhibit a clear tip-focused gradient of H⁺, the pH gradient immediately becomes asymmetrical upon lateral application of gramicidin A, and focal points of acidification are clearly visible on the membrane of the side where the drug was applied.

These data suggest that the absence of Nt AHA in the apex of growing pollen tubes thus produces a sensitized domain that is susceptible to an extra H⁺ influx, which in turn acts by repositioning the growth axis.

Overexpression of Nt AHA-GFP Affects the Physiological Regulation of the Pump and Induces Spatial and Structural Abnormalities on Callose Plug Deposition

With a mechanistic link established between the polarized distribution of Nt AHA and the apical growth of pollen tubes, we decided to gauge the effects of altering either the presence or the activity of the protein. Given the central role of the protein in membrane energization, controlling the levels of expression by knockout or negative-dominant approaches would presumably lead to results as unspecific or difficult to interpret as the ones obtained by pharmacology. Partial modulation of Nt AHA expression by RNA interference would also have been difficult to
interpret, as most RNA interference mechanisms seem to be transcriptionally shut down in Arabidopsis pollen (Pina et al., 2005). We thus decided to screen the transgenic lines stably overexpressing the fusion construct (LAT52:NtAHA-GFP). We reasoned that the presence of GFP in the C terminus of the protein may in some way affect the binding of the 14-3-3 proteins, which seems to be crucial for regulating pumping activity (Palmgren, 2001).

Both transiently transformed and stably transformed (from transgenic plants) pollen expressing Nt AHA-GFP germinated and grew tubes with an apparently normal phenotype under regular germination conditions (pH 5.7). Also, transgenic plants are fully fertile. However, we observed that upon short-time storage at −20°C, transgenic pollen rapidly loses viability. This suggested a possible conditional phenotype either due to overexpression of the H⁺ pump or to an impairment of the pump activity by GFP. This putative phenotype was first investigated regarding pH homeostasis by analyzing both germination rate and pollen tube growth of pollen from the transgenic line versus the wild type germinated at several pH conditions (pH 4, 5, 5.7, 7, and 8). Germination frequency was generally not affected compared with the wild type (Figure 6A), but transgenic pollen tubes consistently showed reduced length, indicating that the growth mechanism was somehow affected.

### Table 1. Effect of Different Gramicidin A Concentrations on Growing Pollen Tubes

<table>
<thead>
<tr>
<th>Gramicidin A Concentration</th>
<th>Effect on Pollen Tube Growth and Features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tube Apex (10 to 15 μm from Tip)</td>
</tr>
<tr>
<td>65 μM</td>
<td>Immediate burst</td>
</tr>
<tr>
<td>30 to 50 μM</td>
<td>Delayed burst (10 to 15 s later) or streaming halted and cytosol pushed forward</td>
</tr>
<tr>
<td>25 μM</td>
<td><strong>Slowdown and reorientation</strong></td>
</tr>
<tr>
<td>20 μM</td>
<td>No effect</td>
</tr>
</tbody>
</table>

**Figure 5.** Application of Gramicidin A at the Lateral Part of the Tip of a Growing Pollen Tube.

(A) to (K) Time-lapse images of pollen tubes challenged with 25 μM gramicidin A. Release of the drug from the micropipette is marked with an arrow. At time 02:20 (min:s), the tube starts to reorient its growth and continues to grow until time 04:30.

(L) Cytosolic pH imaging of a challenged pollen tube. In normal conditions, tobacco pollen tubes show a well-defined acidic tip gradient (inset; Michard et al., 2008), but when challenged with gramicidin, this gradient is visibly delocalized to the side of application, with several acidic spots visible at the point of application (arrows).
Surprisingly, however, we observed that these transgenic pollen tubes produced very abnormal callose plugs with various types of morphology (Figures 6C and 6D). Callose plugs are usually formed as wall ingrowths when the pollen tube reaches a certain length (in tobacco, ~400 to 500 μm), with the aim of isolating the old, nongrowing parts of the tube, which eventually die. However, in Nt AHA-GFP-overexpressing tubes, plugs seem to be deposited only at one side of the tube, and in many cases they fail to close. This abnormal callose plug deposition often results in a phenotype where a series of several incomplete callose plugs allows a continuous cytoplasmic streaming along the entire tube and thus the plugs do not fulfill their ultimate role of isolating the most posterior parts of the tube from the growing tip (see Supplemental Movie 7 online). Finally, while callose plug deposition in the wild type occurs at remarkably similar lengths along the tube (Mascarenhas, 1975), in the overexpressing Nt AHA-GFP tubes, this spatial control was affected.

When measuring extracellular H⁺ fluxes in these abnormal plugs, a series of different flux patterns were obtained, in accordance to the varying morphologies, although it was never similar to the wild type (Figures 6B to 6D). Usually, in cases where a callose plug was being formed only at one side of the tube, there was a very slight decrease in the efflux magnitude at the plug site compared with a neighboring region 10 μm in front of the plug without, however, showing a silent flux as in the wild type (Figure 8). By contrast, on the other side of the tube (where no callose was being deposited), no difference in the efflux profile was detected compared with neighboring regions. In more drastic cases, where the whole tube showed a very small efflux, the neighboring regions closer to the plug presented a slight influx and the site of callose deposition showed a marked influx (Figure 6E).

To assess whether the GFP fusion impaired activity of the pump and thereby resulted in the observed phenotype, germination frequency of both LAT52:NtAHA-GFP and wild-type pollen was measured in the presence of 1 μM fusicoccin, a fungal toxin that irreversibly activates plasma membrane H⁺-ATPases and is known to stimulate pollen germination (Rodríguez-Rosales et al., 1989). Whereas wild-type pollen germination rate is enhanced by ~25% in the presence of fusicoccin, the germination rate of LAT52:NtAHA-GFP seems unaffected by the toxin (Figure 6F). This result suggests that either the chimeric protein is already in an always-active state due to a structural modification of the autoinhibitory C terminus by the GFP fusion, such as what happens when the C terminus of these proteins is truncated (Gevaudant et al., 2007), or the GFP fusion is preventing pump activation via C-terminal phosphorylation and/or binding of 14-3-3 proteins (Palmgren, 2001), which would result in a chimeric pump with reduced or absent activity. It was shown that a similar H⁺-ATPase-GFP fusion protein from N. plumbaginifolia (PMA4-GFP) was unable to complement a yeast mutant strain where the main plasma membrane H⁺-ATPase was deleted (Lefebvre et al.,...
This indicates that AHA is spatially regulated during pollen tube growth (Figure 8). The development of wild-type pollen tubes as a way to assess how Nt AHA-GFP activity supports yeast growth in glucose, producing bigger colonies than AHA2, a wild-type H^+-ATPase from Arabidopsis. The growth of the cells was monitored 3 to 6 d after plating.

**Nt AHA Regulation Predicts Callose Plug Fformation**

Since the ill-formed callose plugs (Figures 6C and 6D) could reflect an impairment of the spatial regulating mechanisms, we decided to investigate the developmental sequence of callose plug formation. Having established that H^+ efflux is a putative marker of Nt AHA, we optimized medium conditions for callose plug formation and closely followed the extracellular H^+ flux profiles with an H^+-selective vibrating probe during the normal development of wild-type pollen tubes as a way to assess how Nt AHA is spatially regulated during pollen tube growth (Figure 8). Early in germination, the hydrated pollen grain shows a H^+-based electric dipole, with an H^+ efflux domain in the entire grain and an influx region at the pore where the tube will grow from, a feature we have established as predictive of tube emergence. As the tube emerges, this dipole is maintained with the influx region now located at the tip of the tube and the most distal parts of the grain showing lower magnitude fluxes than proximal regions (Figure 8A). In 50-μm-long tubes (Figure 8B), the tip-focused influx decreases laterally and a no net flux region appears at 10 to 20 μm from the grain, beyond which point flux is reversed. Up to this point, both influxes and effluxes present approximately the same magnitude (−10 pmol·cm^−2·s^−1). On 100-μm-long tubes (Figure 8C), while the influx region is restricted to the tip, effluxes are now detected along the entire tube and grain except for a subapical transition area spanning ~10 μm. From this point onwards, peak influxes become significantly higher (−20 pmol·cm^−2·s^−1) than effluxes, in some cases by a factor of 4 to 5 times. When the tube is ~200 μm long, the flux pattern observed changes and at the subapical region, behind the tip-focused H^+ influx, a new efflux domain appears, with the previously observed transition area being replaced by a sharp inversion of flux direction, thus originating a current loop at the tip (Figure 8D). This strong efflux region decreases to almost zero flux in the middle of the tube, while a low efflux domain is still present in the grain and the most posterior part of the tube. In tubes longer than 300 μm, this silent domain is progressively and consistently restricted to a more defined spot, ~60 to 80 μm from the grain. Surprisingly, it was consistently observed that this region, observed in 300- to 400-μm-long tubes, is where the first callose plug will be deposited. However, at this length, no structural hint of a plug is yet observable. At this time also, a flux transition occurs in the grain, where the usual efflux domain gives rise first to a null net flux situation (Figure 8F), which then reverts to an influx (Figure 8G) and extends gradually into the posterior part of the tube (Figure 8H) up to the newly formed callose plug (Figure 8I). These fluxes are a clear indicator of the loss of vitality in the older, isolated parts of the tube and the presumable death by acidification. If tubes are followed for longer periods, the same pattern can be observed for each new plug being formed, that is, the position where the plug will be deposited can be predicted by a null net H^+ flux (Figure 8J). Even in tubes longer than 500 μm, the tip region always maintains the short-circuit loop of protons previously formed at 200 μm as long as the tube is growing (Figure 8J). These data show that (1) the positioning of Nt AHA and its exclusion from the tip is strictly regulated and obeys specific spatial relationships but always forms a loop of proton flux through the clear zone at the apical end of the tube; (2) the clearance/inhibition of Nt AHA from specific spots of the membrane where callose plugs will develop is a second spatially regulated feature.

**DISCUSSION**

**Polarization of Proton Fluxes Is Underpinned by an Asymmetric Distribution of Proton Pumps in the Plasma Membrane of Pollen Tubes**

In general, the cytosol of plant cells is kept at a slightly alkaline pH (pH 7.2 to 7.5), while the apoplast is mostly acidic (pH 5 to 6). In pollen from most species, in vitro germination and tube growth are favored by an external acidic environment (pH 5.5 to 6.5) (Linskens and Kroh, 1970). Since the pollen grain was shown to be a major source of outward cationic current (Weisenseel and Jaffe, 1976), to which protons contribute (Feijo et al., 1999), proton pumps are expected to be present in pollen. The observation that germination rate and proton extrusion are enhanced by stimulating H^+-ATPases with fusicoccin and inhibited by blocking H^+-ATPases with orthovanadate (Rodríguez-Rosales...
Figure 8. Spatial and Temporal Profile of Extracellular H⁺ Fluxes in Tobacco Pollen Tubes.
et al., 1989) provided evidence that H⁺-ATPases play a central role in pollen tube growth. Later, an ATPase activity consistent with H⁺-ATPases was shown to be present in pollen grains (Feijó et al., 1992), and these proteins were immunolocalized to the plasma membrane (Obermeyer et al., 1992). Feijó et al. (1999) observed that a proton flux loop was formed at the tip of long pollen tubes (>500 μm) and that this loop coincided with the detected cytosolic pH gradient. While they proposed that H⁺-ATPases could be polarized subapically, they did not rule out alternative scenarios. Assuming that H⁺-ATPases are not polarized, a subapical polarization of regulatory 14-3-3 proteins, which stimulate H⁺-ATPase activity in pollen (Pertl et al., 2001), and/or the apical inhibition of these enzymes by the tip-focused gradient of cytosolic calcium as reported for other plant cells, could account for the fluxes observed. A final hypothesis could simply be that a high concentration of tip-localized proton influx carriers masks the efflux activity of the pumps.

Nt AHA presents homology with some known H⁺-ATPases from Arabidopsis (AHA6, AHA8, and AHA9) and from N. plumbaginifolia (PMA5 and PMA6), which are all pollen-expressed isoforms (Oufatolle et al., 2000; Lefebvre et al., 2005; Pina et al., 2005). AHA9 expression had originally been reported as restricted to anther tissues (Houlé and Boutry, 1994), and recent data for Arabidopsis using DNA microarrays have shown that, together with AHA6, AHA9 expression is pollen specific (Pina et al., 2005). However, despite clear evidence that these enzymes are present in pollen, their role and precise localization remained to be determined.

By expressing Nt AHA cDNA fused with a reporter gene encoding GFP in tobacco pollen, we were able to localize this enzyme in living pollen tubes. In agreement with the previously proposed model for lily pollen tubes (Feijó et al., 1999), we showed that this membrane protein is present in the grain and pollen tube membrane but is excluded from the tip membrane. It appears gradually in the membrane, starting between 10 and 15 μm from the tip. Furthermore, this distribution pattern perfectly fits that of extracellular proton fluxes measured in tobacco pollen tubes. While we cannot ascertain whether this is the only H⁺-ATPase present in tobacco pollen tubes, it is reasonable to assume that the distribution of Nt AHA we show, if not the principal one, may be representative of the class, since there is a strong conservation of their structure. It thus would seem that this differential distribution of H⁺-ATPases along the membrane of growing pollen tubes may constitute one of the molecular mechanisms by which the tip-focused proton loop and consequently the tip-localized pH gradient are established and maintained.

A tip exclusion pattern was also recently observed for another H⁺-ATPase by immunocytolocalization in N. plumbaginifolia pollen tubes (Lefebvre et al., 2005), but the authors also reported that this enzyme is absent at the base of the tube, which is in clear contrast with our results. The possibility that polarization of H⁺-ATPase could be the somewhat nonspecific consequence of the rapid membrane dynamics at the tip was ruled out by a recent report of the localization of a plasma membrane Ca²⁺-ATPase (ACA9) in Arabidopsis pollen tubes, where a yellow fluorescent protein fusion of this pump was shown to accumulate evenly along the membrane, including at the tip (Schiott et al., 2004).

Asymmetric distribution of proton pumps was already known in several highly specialized cells, such as osteoclasts, which use it to produce a localized proton extrusion (Wieczorek et al., 1999). Also recently, asymmetries in the distribution of a V-ATPase and a P-type H⁺,K⁺-ATPase at the tissue level were proposed to be associated with determining left-right patterning in vertebrates (Levin et al., 2002; Adams et al., 2006). Here, we present a polarized distribution of a proton pump as underpinning a current loop associated with growth, in particular with polarized growth.

The subapical alkaline domain resulting from the activity of H⁺-ATPase pumps in this region may have a direct influence on cytoskeleton dynamics, which is crucially involved in polar growth (Kost et al., 2002). Actin-depolymerizing factor (ADF)/cofilin and gelsolin/villin are examples of two candidate pH-dependent direct cytoskeleton modulators. In tobacco and lily pollen tubes, the visualization of actin dynamics using the actin binding protein ADF/cofilin fused to GFP revealed a highly dynamic subapical mesh of short actin filaments with longer actin cables in the shank of the tube (Chen et al., 2002). ADF is known to depolymerize actin more efficiently under alkaline conditions (Hawkins et al., 1993; Maciver et al., 1998) and in the case of the N. tabacum pollen-specific ADF1, this depolymerization was shown to be more efficient in vitro at pH 8.0 than at pH 6.0 (Chen et al., 2002). Moreover, the localization of an actin fringe in lily coincided with the previously determined location of

---

**Figure 8.** (continued).

(A) to (J) Fluxes over time measured at different sites of a particular developmental stage of a growing tube (distances in micrometers are relative to the grain). A schematic representation of the flux pattern is presented for each stage (n > 20 for each stage). Ref, reference measurement; PG1, pollen grain at 180 μm from the tube; PG2, pollen grain at 90° from the tube; PG, pollen grain; INT, interface between grain and tube.

(A) A 10-μm tube presenting H⁺ effluxes in the grain and influx in the emerging tube.

(B) A 50-μm tube showing effluxes in the grain and influxes at the tip and along the tube.

(C) At 100 μm, the influx is restricted to the tip and increases in magnitude considerably, whereas the grain efflux is maintained and spread along the tube up to a flux-silent subapical region.

(D) At 200 μm, a new efflux region appears subapically, while the tip influx is maintained; at ~80 μm from the grain, a new broad flux-silent region appears.

(E) In tubes 300 μm and longer, the tip-localized dipole is maintained; the silent region in the tube becomes spatially more restricted.

(F) to (H) As the tube grows further (to lengths of 300 to 400 μm), a callose plug starts being deposited in the previously detected no net flux region, while the efflux in the grain decreases (F) and eventually reverts to an influx ([G] and [H]).

(I) When the plug is completely formed, the entire region behind the plug shows influxes, and this part of the tube eventually dies.

(J) In tubes longer than 500 μm, new flux-silent regions can progressively be detected in the tube corresponding to sites where callose plugs will form.
the subapical alkaline band, suggesting an obvious role for pH in actin turnover (Cárdenas et al., 2005; Lovy-Wheeler et al., 2006). Gelsolin is an actin binding protein that/severs preexisting actin filaments and caps them, thereby preventing further polymerization (McGough et al., 2003). This activity is known to strongly depend on Ca\(^{2+}\) (Khältina and Hinssen, 2002), but several data point to an alternative activation of gelsolin by low pH that, to some extent, overrides the requirement for Ca\(^{2+}\) (Lamb et al., 1993; Lagarrigue et al., 2003). Gelsolin is known to be present in pollen and colocalizes with actin bundles (Vidali et al., 1999), in the germinal furrows of the grain, and in the tube cytosol, mainly in the tip (Tao and Ren, 2003). It was proposed that the intracellular tip-focused gradient of [Ca\(^{2+}\)] could be involved in its activation (Vidali et al., 1999), but considering that pollen tubes also present an acidic tip, it is tempting to think that a low pH may also contribute to the absence of actin polymerization in this region, further reinforcing the idea that the remodeling of the actin cytoskeleton could be modulated along a pH gradient.

In an even more speculative hypothesis, this pH modulation could also be achieved indirectly by a pH dependence of protein phosphatases that would act on direct modulators of the cytoskeleton, like ADF/cofilin (Chen et al., 2003) or small GTPases (Kost et al., 1999). It is known that pollen tube growth is affected by reversible protein phosphorylation and that this mechanism involves the cytoskeleton (Obermeyer et al., 1998; Foissner et al., 2002). Evidence from crawling nematode sperm cells where a cytoplasmic pH gradient regulates gelation and solation of cytoskeleton fibers along the lammelipod (Italiano et al., 1999) reveals that pH does not trigger contraction directly but may strongly contribute to the activation of endogenous phosphatases that would participate in the disassembly of the fiber bundles (Miao et al., 2003).

### Proton Pumps Do Not Freely Diffuse in the Plasma Membrane

Techniques for labeling proteins with fluorescent tags like GFP (Prasher et al., 1992; Chalfie et al., 1994) have made it possible to follow protein dynamics in living cells, including its formation, localization, and kinetics. In parallel and as a consequence, imaging techniques like FRAP have been developed to fully explore the information provided by expression of GFP fusion proteins. Proteins inside cells are either embedded in, or associated with, membranes, or they are free in an aqueous phase like the cytoplasm, the nucleoplasm, or organelle lumen. Within each of these environments a protein can either freely diffuse, be immobilized to a scaffold or be actively transported. A way to assess these mobility properties is by using FRAP and calculating the diffusional mobility through the diffusion coefficient or by particle tracking.

The mobility of Nt AHA-GFP was assessed in pollen tubes via FRAP and particle tracking. These proteins were seen to move directionally toward the tip with a mobility of 0.2 to 0.5 \(\mu\)m/min (0.003 to 0.008 \(\mu\)m/s). These values are 100-fold lower than the ones reported for other membrane proteins known to freely diffuse in the plasma membrane, such as in the case of another P-type ATPase, an Na\(^+\)-K\(^{+}\)-ATPase, in low-density MDCK cells (Jesatis and Yguerabide, 1986) and E-cadherin in epithelial cells before adhesion (Adams et al., 1998). This difference argues for a tethering in the mobility of Nt AHA-GFP since, unlike free diffusion, the movement is directional and therefore active. An obvious hypothesis for this structural property would be an active involvement of the cytoskeleton in this process as reported for membrane proteins in other systems (Nakada et al., 2003).

### How Do Proton Pumps Get Polarized?

Although all growing Nt AHA-GFP expressing tubes showed a V-shaped apical intracellular labeling pattern colocalizing with a region known to be rich in secretory vesicles that deliver cell wall material to the tip (Steer and Steer, 1989), it is hard to conceive that Nt AHA also follows this apical exocytic route since no labeling is seen in the membrane tip. Then the questions arise: where are these H\(^{+}\) pumps being inserted in the membrane and what is driving their polarization?

From the FRAP results we cannot say precisely where these proteins are being inserted. Yet, as they appear to be moving in the membrane toward the tip, this would strongly argue that either they may be specifically retrieved from the membrane at the subapical region or otherwise there is a constant pool of H\(^{+}\)-ATPases moving with growth without any membrane turnover. Supporting evidence for the first hypothesis comes from reports of apical endocytosis in pollen tubes (Parton et al., 2001, 2003). Endocytic and exocytic vesicles colocalize at the tip, and dynamic membrane traffic is observed at that region, which would explain the intracellular apical labeling. Moreover, it was also shown in yeast that a slow diffusion of certain proteins in the membrane (diffusion coefficient of 0.0025, in the same order of magnitude as the mobility of Nt AHA-GFP) allows for polarity to be maintained only by endocytic cycling (Valdez-Taubas and Pelham, 2003), a concept that had already been pointed out as a simple general method for generating cell asymmetry (Bretscher, 1996). The second hypothesis, in which H\(^{+}\)-ATPases would accompany growth, would have to involve a diffusion barrier preventing these proteins from entering the tip region. Diffusion barriers in the plasma membrane have been found in many cell types and are known to be involved in regions of polarization, such as the involvement of septin in the neck of the growing bud in budding yeast (Takizawa et al., 2000) and the boundary between anterior and posterior tail domains in mammalian sperm (Bartles, 1995). Also, in axons, the creation of rows of densely packed anchored protein pickets was proposed as a general strategy for forming such diffusion barriers (Nakada et al., 2003). As a complement, Nt AHA may be localized in membrane lipid microdomains (or rafts). In yeast, the H\(^{+}\)-ATPase PMA1 was shown to be delivered to the plasma membrane in association with lipid microdomains (Bagnat et al., 2001). In contrast with animal cells, this association in yeast happens at the ER and is needed both for oligomerization of the enzyme monomers and for its stability at the plasma membrane (Wang and Chang, 2002). Lipid microdomains were also reported to provide specific endocytic signals for certain proteins (Simons and Ikonen, 1997; Sharma et al., 2002).

Our results from the in situ hybridization showed that Nt AHA mRNA was absent from the tip region. This further suggests that Nt AHA is probably being inserted in the plasma membrane everywhere but in the tip using a general exocytic pathway.
different from the tip-targeted pathway used for cell wall material, as would happen in a diffuse growth system. H\textsuperscript{+}-ATPase mRNA levels have been estimated at 2,500 to 10,000 molecules per cell in lily pollen protoplasts by single-cell RT-PCR (Gehwolf et al., 2002). However, this previous study only revealed that there was no correlation between the levels of pump activity and the levels of mRNA, confirming that the pumps are posttranslationally regulated. The submembrane detection of Nt AHA mRNA in this study further suggests that these proteins may be delivered to the plasma membrane directly from the ER, which would explain why Nt AHA-GFP labeling is almost absent inside the tube. This would not be surprising considering that pollen tubes were previously reported to have ER closely appressed to the membrane with no space between them (Lancelle and Hepler, 1992). Also, a close association of the ER with microtubules and microfilaments has been reported in Nicotiana alata pollen tubes (Lancelle et al., 1987). This further supports the idea that Nt AHA could be delivered to the plasma membrane in ER-born membrane domains that would then be driven toward the tip by the cytoskeleton.

**The Role of Nt AHA Polarization in the Definition of the Growth Axis in Pollen Tubes**

Cellular polarization is crucial for many biological processes, including cell morphogenesis, proliferation, and differentiation. The orientation of the polarity axis is initially defined by asymmetrical extrinsic or intrinsic cues acting at the cell surface, which then have to be recognized and interpreted by signaling molecules, leading to the asymmetrical activation/inhibition and/or distribution of downstream effectors. This asymmetry is further stabilized by rearrangements of the cytoskeleton, enabling the cell to assume an asymmetric shape (Sohrmann and Peter, 2003).

In pollen tubes, we can consider two distinct axes: the polarity axis and the growth axis. The polarity axis can be drawn transversally at the base of the tip dome separating the tip region from the rest of the tube, whereas the growth axis is perpendicular to the polarity axis and divides the tubes longitudinally (symmetry axis). Despite the fact that pollen tubes are highly polarized cells undergoing polar growth, they have a very different polarity mechanism from other systems, such as Fucus zygotes, where an asymmetrical division establishes the axis of polarity without any external cue (Brownlee and Bouget, 1998). On our assumption that protons can significantly contribute to the definition of polarity in this system, the asymmetrical distribution of H\textsuperscript{+} pumps observed could be regarded as an endogenous mechanism for establishing the polarity axis giving rise to the tip-localized pH gradient that would in turn contribute to the definition of the growth axis.

Reorientation responses have previously been obtained in pollen tubes after photoactivated local release of caged calcium in the flanks of the apical dome (Malho and Trewavas, 1996). These experiments resulted in lateral reorientation toward the side of release, and this suggested a role for calcium in controlling the position of the growth axis by controlling apical exocytosis (Malhô et al., 1995). By applying gramicidin A externally and laterally at the tip dome, we obtained a similar yet faster reorientation response than with calcium. Gramicidin A forms water-filled pores that continuously conduct monovalent cations through a water-wire mechanism (Hille, 2001). In the specific growth conditions used to germinate pollen, two monovalent ions are present that could presumably trigger currents through the gramicidin A pore, H\textsuperscript{+} and K\textsuperscript{+}, both of which have a concentration gradient across the plasma membrane of roughly two orders of magnitude. It should, nevertheless, be mentioned that the permeability for H\textsuperscript{+} is 12.8 times higher than for K\textsuperscript{+}, thus allowing the assumption that this will be the ion that will occupy the pore more efficiently. One cannot, however, overrule some K\textsuperscript{+} transport, which could presumably lead to a membrane depolarization. However, this effect would immediately be propagated to the whole cell and would be independent of the position where gramicidin A was applied, making it hard to explain the localized and polarized effect gramicidin A induces. Imaging the pH effectively showed that gramicidin A induced a lateral polarization of cytosolic pH, with discrete spots of acidification, which presumably correspond to the local patch contact of the cell wall pores after application. One may thus conclude that the effect of gramicidin A is largely due to H\textsuperscript{+} movement through the pore.

Although we applied gramicidin A at several sites (at the tip, subapically, in the shank of the tube, and in the grain), a reorientation response only occurred when we applied it laterally at the tip dome within 5 to 10 \(\mu\)m from the apex. A possible explanation could simply lie on the kinetics of the pore since in liposomes the gramicidin A channel is known to be mechanically activated/inactivated through changes in the thickness of the lipid bilayer (Martinac and Hamill, 2002). However, we think this hypothesis can be ruled out in this system given the lethal responses obtained upon application of higher concentrations of the drug in all regions of the pollen tube and grain. Upon insertion of the drug in the membrane and consequent pore formation, protons will rapidly diffuse inside the cell, which will create a new submembrane acidic domain. The response to gramicidin A is quick and continuous, suggesting a direct mechanistic effect. Given the known tip-focused pH gradient observed in pollen tubes (Feijô et al., 1999), the spatially restricted response to gramicidin A points to an important function for proton signaling in defining growth orientation: slight changes inside the pH gradient will be transduced into reorientation responses, whereas changes outside this gradient and therefore outside the tip region will not be perceived as a signal. The differential localization of the H\textsuperscript{+} pumps makes it possible for an apical pH sensing machinery to exist.

The putative role of the tip-focused acidic domain in promoting exocytosis (Feijô et al., 1999), and these results now strongly support the idea that either exocytosis or endocytosis may be a downstream target of pH signaling. The shift in the position of the acidic domain after gramicidin application can also be seen as producing a vectorial response of the entire gradient, as happens with the cAMP gradient in Dictyostelium (Marée, 2000), thus creating a deformation of the pH waves that tends to self-restore. Interestingly, in all cases, the reoriented growth was not sustained for a long time, with pollen tubes permanently stopping after some 20 \(\mu\)m of growth in the new direction. Also, attempts of secondary applications of the drug at the opposite side resulted in immediate growth arrest or tube bursting. One possible explanation lies in the fact that gramicidin A channels, once inserted, are always open and nonregulated. As the tube grows they will be pushed...
backward in the tip membrane and will reach a point where they will colocalize with the subapical H\(^+\) pumps, thus short-circuiting the proton energization and ultimately leading to an acidification of the cytosol and consequent death.

Although a great deal of controversy still exists on a possible signaling role for pH gradients due to the extremely high conductivity of protons with consequent dissipation of transiently formed gradients, the results presented here constitute strong evidence for pH gradient–based proton signaling operating at the tip of growing pollen tubes. Furthermore, we believe protons may also act as long-distance signals over a time frame too short to be resolved by current techniques. Protons move in aqueous solutions using hydrogen bonds by the so-called Grothuss mechanism (i.e., a proton added to a water molecule or other hydroxyl-containing molecule makes the proton from the opposite side of the bond where the new proton was added hop to the next molecule, which will produce the same effect on the opposite proton and so on) (DeCoursey, 2003). This makes protons the only known ion that can propagate in space in a virtual way (i.e., by changes in the identity of the molecules that participate in the hydrogen-bonded network). This same property has recently been proposed to be at the basis for the propagation of fast calcium waves, which would be preceded and driven by a proton wave (Jaffe, 2004). In the case of pollen tubes, this type of signaling would be of utmost importance in continuously establishing the polarity axis and in the constant feedback required for stabilizing polarity through, for example, dynamic rearrangements of the cytoskeleton.

While the functional significance of the apical closed proton loop is yet to be determined, it was proposed that it might be a property that would allow the pollen tube to perceive and respond to female signals by instantly modifying structural pH-sensitive targets and consequently redirecting its growth (Feijo et al., 1999). It is known that pollen tubes grow parallel to an applied field and that this orientation is more influenced by certain ions like chloride (Malho et al., 1992b). It therefore seems plausible that this apical field may be used by the pollen tube to sense small external conductance heterogeneities that would then be instantly transduced into reorientation responses, possibly via a pH-sensing mechanism.

Taken together, our results suggest that proton dynamics may play an important role in the establishment and maintenance of polarity of apically growing cells. Furthermore, we showed that the definition of the apex as the point of growth has a strong molecular basis in which the exclusion of a proton transporter generates a special intracellular environment that renders it susceptible to proton-modulated signaling, in particular regarding the definition of the growth axis. This work thus provides insights into a general mechanism in which ion dynamics, and in particular proton dynamics, could act as developmental switches involved in cellular growth and patterning.

**METHODS**

**Plant Material**

Tobacco (*Nicotiana tabacum* cultivars Petit Havana SR1 and Samsung) plants were grown under greenhouse conditions until flowering for pollen collection and cultured in vitro for plant transformation. Pollen used in all assays was collected from anthers immediately after anthesis and used fresh or frozen at –20°C.

**Pollen Germination and Pharmacology**

Tobacco pollen grains were germinated in S Medium (1.6 mM H\(_2\)BO\(_3\), 200 \(\mu\)M CaCl\(_2\), 6% sucrose, and 1 mM MES, pH 5.7).

For proton flux measurements, this medium was modified to contain only 25 \(\mu\)M MES, and measurements were made on tubes of 0 to 600 \(\mu\)m in length, with growth rates ranging from 1.2 to 2.5 \(\mu\)m min\(^{-1}\) and a culture density of 0.06 mg mL\(^{-1}\). For pharmacological assays, pollen was used at a final culture density of 1.0 mg mL\(^{-1}\). Pollen tubes were allowed to grow at room temperature in the dark for between 3 h and overnight, depending on the assay, with gentle shaking, and when appropriate, fixed with Calcium-Baker fixative (Baker, 1946). For phenotype determination, both transgenic (LAT52:NTAHA-GFP) and wild-type pollen were germinated in S Medium at varying pH (4, 5, 5.7, 7, and 8) for 3 h, and average germination rates and pollen tube lengths were determined for each pH condition.

In the growth inhibition assays, the inhibitors sodium orthovanadate (Sigma-Aldrich), concanamycin A (Sigma-Aldrich), N-ethylmaleimide (Sigma-Aldrich), and sodium azide (Merck) were used at several concentrations within the micromolar range. The length of 100 pollen tubes was measured for each concentration, and an IC\(_{50}\) was determined for each inhibitor. In flux measurement assays, inhibitors were added to the pollen culture after a pretreatment flux profile had been obtained for 100-\(\mu\)m-long tubes. Flux data collection was then resumed and respective growth rates recorded.

Fusicoccin was used at a final concentration of 1 \(\mu\)M as previously described (Pertl et al., 2001).

Gramicidin A (Fluka) was kept as a stock solution of 130 mM in DMSO.

Dose–response curves for pollen tube growth were obtained using 0 nM, 5 nM, 10 nM, 50 nM, 500 nM, 1 \(\mu\)M, 10 \(\mu\)M, 50 \(\mu\)M, and 100 \(\mu\)M gramicidin A in germination medium. Pollen was incubated in S Medium for 1 h before adding the drug and was incubated for another 1.5 h before fixation with Calcium-Baker fixative (Baker, 1946). One hundred pollen tubes were measured for each concentration. To create artificial gradients of gramicidin A, glass capillaries of 1.5 mm external diameter were pulled into pipettes with an external tip diameter of \(\sim\)6.5 \(\mu\)m using a Flaming-Brown micropipette puller (model P-97; Sutter Instrument). The pipette tips were filled by direct tip submersion in 1% low-melting-point agarose (Sigma-Aldrich) containing several concentrations of gramicidin, ranging from 100 \(\mu\)M to 1.3 mM, and placed close to growing pollen tubes using a micromanipulator (model MMO-203; Narishige). For in situ application, glass capillaries of 1.0 mm external diameter (World Precision Instruments) were pulled into pipettes with an external tip diameter of \(\sim\)3 to 4 \(\mu\)m. The tips were backfilled with several concentrations of gramicidin A in DMSO (13 to 65 \(\mu\)M), and the pipette was topped off with water, fitted onto microinjector tubing (Eppendorf), and placed close to growing pollen tubes at different positions. The pipette was then placed in contact with the cell wall of the tube and an amount of drug was pressure-released. In most cases, the drug was clearly visible due to an immediate change of phase upon contact with the aqueous medium. Images were collected every 2 s.

**Proton Flux Measurements**

An ion-selective vibrating electrode (Kühntreiber and Jaffe, 1990; Shipley and Feijó, 1998; Feijó et al., 1999) was used to measure extracellular H\(^+\) fluxes in pollen tubes generally as described by Zonia et al. (2002). To build the H\(^+\)-specific electrode, 1.5-mm borosilicate glass electrodes (tip diameter 1 to 3 \(\mu\)m) were covered with N,N-dimethyltrimethylhexylamine (Fluka), backfilled with a 10- to 20-mm column of 40 mM KH\(_2\)PO\(_4\)/15 mM KH\(_2\)PO\(_4\).
KCl, pH 6.0, and front-loaded with a 25-μM column of a H+selective liquid exchange cocktail (Hydrogen Ionomophore Cocktail B; Fluka). A dry reference electrode (DryRef-2; World Precision Instruments) completed the measuring circuit. Electrode calibration was done by measuring the nernstian potential of three solutions of pH 7, 6, and 4 (H+ 10-4, 10-3, and 10-1, respectively). The vibrating electrode system was used as previously described (Shipley and Feijó, 1998; Zonia et al., 2002). Briefly, signals were measured with a purpose built electrometer (Applicable Electronics). Electrode vibration and positioning was achieved with a stepper motor-driven three-dimensional positioner. Data acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper motor–controlled fine focus of the microscope stage were performed with ASET software (Science Wares and Applicable Electronics). The self-referencing vibrating probe oscillated with an excursion of 10 μm, completing a whole cycle in 2 s, including a tunable setting time after each move, two measure periods (one at each extreme) and the excursion time. The measurement close to the membrane was then subtracted from the one further away. This subtraction represents the self-referencing feature of the probe. Fluxes at the surface of a pollen tube were measured by one-directional vibration of the electrode tip as close as possible to the perpendicular (90°) of the tube surface without touching the tube. The extreme measuring point was positioned to be within 1 to 3 μm of the tube surface where fluxes are assumed to be uniform (Smith et al., 1994). Background references were taken at >500 μM away from any pollen grain or tube, and the values were subtracted from the millivolt differential recordings during data processing using Microsoft Excel 2000. Fluxes were calculated using Fick’s law and the proton diffusion coefficient in aqueous solution at 25°C (9.37 × 10-6 cm2·s−1) (Weast, 1987).

Isolation and Cloning of Nt AHA cDNA

All molecular cloning protocols were performed in accordance with Sambrook and Russell (2001). Oligonucleotide sequences show the nucleotides corresponding to added restriction sites (underlined) with the respective restriction enzyme at the end. All restriction enzymes were from New England Biolabs.

A 1460-bp fragment inside the Arabidopsis thaliana aha1 gene was amplified by RT-PCR from 5 μg of poly(A)+ RNA of young flowers of Arabidopsis ecotype Columbia, using 10 pmol of each oligonucleotide (5'-GGCGATCCGAGAAAACAAAAGAAAGCCCAGGTGG-3', BamHI; 5'-GCGGATCCGAGAAAACAAAAGAAAGCCCAGGTGG-3', EcoRI; 5'-GCGGATCCGAGAAAACAAAAGAAAGCCCAGGTGG-3', SalI). The PCR product was blunt-end ligated into previously EcoRV-digested pBluescript KS− (Stratagene) and cloned in DH5α competent cells. This C-terminal fragment was 32P-radioabeled and used as a probe to screen a previously generated λ cDNA library from mature pollen of tobacco (Chen et al., 2002) at a hybridization temperature of 58°C. The pBluescript phagemid was excised according to the protocol in the λZAPII library construct kit (Stratagene), and putative full-length cDNAs were sequenced in an automated DNA sequencer ABI377 (Applied Biosystems). The sequence was further analyzed using the software package VectorNTI (InforMax). Further sequence and homology analysis and predictions were done using publicly available software (ProtParam, Prosite, PSIPRED v2.4, TopPredII, and BLAST).

Phylogenetic Analysis

Alignment of Nt AHA, Arabidopsis AHas, and Nicotiana plumbaginifolia PMAs was done using ClustalW (Thompson et al., 1994) with the following parameters: gap open penalty = 10; gap extension penalty = 0.05; weight matrix = BLOSUM (for protein). From this alignment, an unrooted dendrogram was produced.

Expression Construct

A full-length cDNA (Nt AHA) obtained from the pollen library was subcloned into a pBluescript SK+–based expression vector containing an engineered GFP cDNA (Chiu et al., 1996) under the control of the pollen-specific promoter LAT52 (Twell et al., 1990) for pollen expression (pLAT52:NcoI-GFP). Nt AHA cDNA was introduced in frame at the 5′ end of GFP using a 5′ BamHI site in the cloning site of ZAPII and the C-terminal oligonucleotide 5′-GCGGATCCGAGAAAACAAAAGAAAGCCCAGGTGG-3′ (NcoI) to generate a 3′ NcoI site by PCR. The resulting expression construct (LAT52:NHA-GFP) was either used directly for transient expression in pollen or inserted into an intermediate Ti plasmid vector for plant transformation.

To generate the catalytically dead Nt AHA-GFP, site-directed mutagenesis was performed in the LAT52:NHA-GFP construct using the forward primer 5′-GGATGTCTTTGCAGTAACAAAGACCGGTACC-3′ and its complementary reverse primer to substitute a single nucleotide (underlined) (QuickChange kit; Stratagene).

Pollen Bombardment

Ten milligrams of mature fresh or frozen pollen grains were used in each bombardment. Just before bombardment, pollen was suspended in S medium and spotted on the center of 35-mm Petri dishes containing semisolid S Medium with 0.7% agarose. After bombardment, pollen grains were transferred to glass-bottom Petri dishes previously coated with 0.01% poly-l-lysine (Sigma-Aldrich) and allowed to grow for ~4 to 5 h at room temperature in the dark.

Microparticle bombardment was performed using the PDS-1000/He biolistic system (Bio-Rad). Gold particles (1.0 μm diameter) were coated with the DNA according to the manufacturer’s instructions (Bio-Rad; Sanford et al., 1993). Approximately 5 μg of DNA coated ~3 mg of particles. These were used for two bombardments per sample to increase the transformation frequency. The parameters of bombardment were as follows: 700 mm Hg chamber vacuum, 1100 p.s.i. rupture disc, 0.625-cm gap distance, and 2.5-cm particle travel distance. As a control for transformation and for assessment of the transgene expression level, bombardment was done using LAT52:NcoI-GFP alone.

Plant Transformation

Tobacco leaf disc transformation by Agrobacterium tumefaciens Ti plasmid was performed according to a standard protocol (Delebreze et al., 1986). The Ti plasmid containing the GFP chimeric gene was first transferred from Echerichia coli DH5α into Agrobacterium strain GV2260 by conjugation.

Plants were grown in vitro in plant medium (per liter, 100 mL × Gamborg’s B5 salts [Sigma-Aldrich], 1 mL 1000× B5 vitamins [Sigma-Aldrich], 0.5 g myo-inositol, 100 mg MES buffer, 10 g sucrose, pH 5.7, and 7 g agar) for ~6 to 8 weeks. Agrobacterium carrying the construct was inoculated in 3 mL Min A medium (per liter, 6.99 g K2HPO4, 3H2O, 2.25 g KH2PO4, 0.5 g (NH4)2SO4, 0.25 g Na-citrate-2H2O, 1 g glucose, 0.05 g MgSO4·7H2O, and 0.0025 g B1 vitamin) and grown for 24 h at 30°C. Approximately 90 leaf discs were used for infection (150 μL bacterial culture). Shoot formation was induced in shoot-inducing medium (per liter, 100 mL × Gamborg’s B5 salts [Sigma-Aldrich], 1 mL 1000× B5 vitamins [Sigma-Aldrich], 0.5 g myo-inositol, 100 mg MES buffer, 30 g sucrose, pH 5.7, 7 g agar, 1 mg bis-aminopiridine, 0.1 mg indolacetic acid, and 250 mg cefotaxime), and transformants were selected with 50 mg/L kanamycin. Plantlets were then transferred to soil in greenhouse conditions, and, upon flowering, pollen was collected and imaged. Transgenic M1 plants were grown on soil, and pollen was collected for pH and pharmacological assays.

Microscopy and Image Analysis

Fluorescence imaging of pollen tubes was done using two-photon microscopy (reviewed in Feijó and Moreno, 2004) in a Bio-Rad 1024MP,
Nikon TE-300, PlanFluo optics (×40 numerical aperture 1.3, oil); for excitation, we used the Coherent Mira-Verdi Ti-Sapphire laser at 870 to 90 nm (for GFP) and 802 nm (for AlexaFluor-488), pulse width < 150 fs. Bright-field imaging was done on a Nikon TE-300 microscope equipped with a cooled CCD camera (Princeton Micromax 5 MHz). Bright-field image acquisition and analysis were performed using the software package Metamorph v.6.1 (Universal Imaging Corporation).

Fluorescence bleaching (FRAP) in both transiently transformed and stable transgenic pollen tubes expressing the LAT52:NtAHA-GFP construct was done at ~50 μm from the tip, using the following settings on the two-photon microscope: zoom 50, 16% laser power, 6× scan. Subsequent time-lapse sequences were acquired with a 10-s interval between frames. Analysis on the recovery of fluorescence kinetics was done using the track object function of the software package Metamorph v.6.1.

In pollen tubes transiently expressing Nt AHA-GFP, the average pixel intensity along the GFP-labeled borders was measured using the linescan measurement function of the software Metamorph v.6.1, producing a table with pixel intensity versus distance to the tip. Statistical analysis of the data was done using a custom-made code (J. Carneiro, Instituto Gulbenkian de Ciência). Briefly, data series were smoothed by moving average procedure and then detrended by taking the difference between the original series and the smoothed series. Lomb-Scargle periodograms (i.e., spectral analysis of unevenly sampled data) (Press et al., 1992), were performed on original, smoothed, and detrended series. Periods with power values whose probability of being due to white noise was lower than P < 0.01 were considered significant.

Cytosolic pH imaging was done as described by Michard et al. (2008). In short, we used the ratiometric form of the genetically modified pH-sensitive GFP, pHluorin (Miesenbock et al., 1998) under the LAT52 promoter, and transformed pollen as described above. Imaging was done in a wide-field system (Nikon Eclipse, S-Fluo lens, ×40, numerical aperture of 1.3) using the bottom port and a Princeton Micromax cooled CCD. Excitation was produced by a Sutter DG-4 by consecutive excitation with pixel intensity versus distance to the tip. Statistical analysis of the data was done using the track object function of the software package Metamorph v.6.1.

Subsequent time-lapse sequences were acquired with a 10-s interval for 24 consecutive time frames. Data series were then smoothed by moving average procedure and then detrended by taking the difference between the original series and the smoothed series. Lomb-Scargle periodograms (i.e., spectral analysis of unevenly sampled data) (Press et al., 1992), were performed on original, smoothed, and detrended series. Periods with power values whose probability of being due to white noise was lower than P < 0.01 were considered significant.

Whole-Mount in Situ Hybridization

Three different fragments of Nt AHA were subcloned into pBluescript SK+ (Stratagene) to generate sense and antisense RNA probes by in vitro transcription. Plasmids were linearized with appropriate restriction enzymes and in vitro transcribed following the protocol proposed by Molecular Probes. Briefly, a reaction containing 1.0 μL each 10 mM ATP, 10 mM CTP, 10 mM GTP, 0.75 μL of 10 mM UTP (Roche), 2.5 μL of 1 mM ChromaTide UDP-AlexaFluor488 (Molecular Probes), 2 μL of 10× transcription buffer (Roche), 1.0 units of RNasin (Promega), ~500 ng DNA template, 2 to 4 units of T3 or T7 RNA polymerase (Roche), and RNase-free water up to a final volume of 20 μL was incubated for 2 h at 37°C. Then, 10 units of RNase-free DNase I (Ambion) were added, and the reaction was incubated for 15 min at 37°C. The reaction was stopped by addition of 2 μL of 0.2 M EDTA, pH 8.0 (Sigma-Aldrich). RNA was purified using Micro Bio-Spin Columns P-30 (Bio-Rad) following the manufacturer’s instructions with Tris buffer replaced by diethylpyrocarbonate-treated water. Probes were then fragmented into 40- to 150-bp fragments with 1× fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, and 150 mM MgOAc) for 30 min at 95°C, and aliquots of 2 μL were taken for analysis in an Agilent 2100 Bioanalyzer, running Bio sizing software version A.02.12 and using an RNA 6000 Nano LabChip andreg kit (Agilent), following the manufacturer’s instructions.

All hybridization procedures were done either in 1.5-mL centrifuge tubes with quick spins between washes or using a vacuum-driven dot blot apparatus from Bio-Rad with a 10-μm pore diameter membrane. Tobacco pollen was germinated in S medium for 3 h and fixed in 2% paraformaldehyde overnight at 4°C. It was then dehydrated in an increasing series of methanol (25%, 50%, 75%, and 100% in PBS), rehydrated in a decreasing series of methanol (100%, 75%, 50%, and 25% in PBS), and washed twice in PBS. It was incubated for 30 min with 1 μg/mL of proteinase K (in PBS; Roche) and 5 min in glycine (2 mg/mL in PBS; Sigma-Aldrich), both at room temperature, and finally washed twice in PBS. Prehybridization was performed for 2 h at 55°C in hybridization buffer (50% formamide [Roche], 5× SSC, 50 μg/mL tRNA [Sigma-Aldrich], 50 μg/mL heparin [Sigma-Aldrich], and diethylpyrocarbonate-water) and subsequently hybridization took place at 55°C overnight with ~500 ng of probe in the hybridization buffer. Controls were done using the sense probe and with no probe to check for autofluorescence.

Overnight samples were washed for 2× 60 min at 55°C in a solution containing 20 mL of formamide (Roche), 8 mL of 20× SSC, pH 4.5, 8 mL 10% SDS, and 4 mL sterile water and then washed for 2× 30 min at 55°C in a solution containing 20 mL of formamide (Roche), 4 mL of 20× SSC, pH 4.5, and 16 mL of sterile water. Pollen samples were finally washed 2× in PBS, resuspended in 200 μL glycerol/PBS (9:1), and mounted between slide and cover slip with ProLong Antifade (Molecular Probes) before microscopic observation.

Yeast Complementation Assays

A BamHI-Sal fragment from LAT52:NtAHA-GFP containing Nt AHA-GFP was cloned blunt-ended into a modified yeast expression vector based on pMP136. pMP136 was digested with Xhol and Spel to remove AHA2, dephosphorylated, and blunt-ended with Klenow. After ligation and transformation into E. coli Stbl2 competent cells (Invitrogen), the resulting expression vector pYENT AHA-GFP was amplified and transformed into Saccharomyces cerevisiae strain RS-72 (Mat a; ade1-100 his4-s19 leu2-3, 312 pMA1-pGAL1) to be used for complementation tests according to Buch-Pedersen and Palmgren (2003). Selected yeast transformants were confirmed by GFP labeling at the plasma membrane (see Supplemental Figure 8 online). Each complementation experiment was replicated independently four times. In drop tests, cells were diluted in sterile water and 5 μL were spotted at two concentrations (OD600 = 1 and 0.1) on selective media, pH 5.5, 4.5, and 6.5. The original pMP136 was used to express native AHA2.

Accession Numbers

Sequence data from this article can be found in the GenBank and Arabidopsis Genome Initiative databases under the following accession numbers: Nt AHA (AY383599), AHA1 (AAA32813), AHA2 (P19456), AHA3 (P20431), AHA4 (Q95SU5), AHA5 (AAD23893), AHA6 (NP_178762), AHA7 (T49228), AHA8 (T47332), AHA9 (Q42556), AHA10 (NP_173169), AHA11 (Q9LV11), PMA1 (Q08435), PMA2 (Q42932), PMA3 (Q06436), PMA4 (Q03194), PMA5 (AAV49166), PMA6 (Q9SWH2), PMA8 (Q9SWH1), and PMA9 (Q9SWH0).

Supplemental Data

The following materials are available in the online version of this article:

Supplemental Figure 1. Effect of Four Different H+ Pump Inhibitors on Extracellular H+ Fluxes at Five Distinct Regions of Tobacco Pollen Tubes.

Supplemental Figure 2. Membrane Region Prediction for Both Nt AHA and AHA1.

Supplemental Figure 3. Unrooted Dendrogram of Nt AHA, Arabidopsis AHAs, and N. plumbaginifolia PMAs.
The Plant Cell

Supplemental Figure 4. Transient Expression of a Catalytically Dead Nt AHA-GFP and Wild-Type Nt AHA-GFP in Tobacco Pollen Tubes.

Supplemental Figure 5. Dose–Response Curve for Gramicidin A in Growing Tobacco Pollen Tubes.

Supplemental Figure 6. Control Experiment with the Application of DMSO Laterally at the Tip of a Pollen Tube.

Supplemental Figure 7. Yeast Complementation to Assess Individual Colony Size.

Supplemental Figure 8. GFP Labeling of Yeast Cells Transformed with Nt AHA-GFP

Supplemental Table 1. Population Effects for the Four Major Proton Pump Inhibitors Assessed in Pollen.

Supplemental Movie 1. Growing Pollen Tube Expressing Nt AHA-GFP.

Supplemental Movie 2. FRAP in the Membrane of a LAT52:NtAHA-GFP Pollen Tube.

Supplemental Movie 3. Application of 45 μM Gramicidin A to a Pollen Tube.


Supplemental Movie 5. Application of 25 μM Gramicidin A to a Pollen Tube Laterally at the Apical Dome.


Supplemental Data Set 1. Amino Acid Sequence Alignment Used to Generate.

ACKNOWLEDGMENTS

We thank Michael Palmgren for having kindly offered the yeast expression vector and the yeast strain RS-72. We also thank Miguel Godinho and Clara Reis at the Instituto Gulbenkian de Ciência for technical advice and Engº Margarida Teixeira Santos at the Estação Agronômica Nacional for providing Samsum tobacco plants. This work was supported by the Fundação para a Ciência e Tecnologia (POCTI/34772/BCI/2000, POCTI/BIA-BCM/60046/2004, and PPCDT/BIA-BCM/61270/2004), the National Institutes of Health (HG52953), U.S. Department of Energy (97ER20288), and the USDA (0101936). A.C.C. acknowledges Fundación Luso-Americana for providing Samsun tobacco plants. This work was supported by grants from the Foundation for Science and Technology (POCTI/BD19874/1999 and POCTI/BPD14697/2003) and PPCDT/BIA-BCM/61270/2004, and the FCT Fellowships (POCTI/BD19874/1999 and POCTI/BPD14697/2003) and a Fundação Luso-Americana para o Desenvolvimento travel grant.

Received September 18, 2006; revised February 13, 2008; accepted February 29, 2008; published March 25, 2008.

REFERENCES


Exclusion of a Proton ATPase from the Apical Membrane Is Associated with Cell Polarity and Tip Growth in *Nicotiana tabacum* Pollen Tubes

Ana C. Certal, Ricardo B. Almeida, Lara M. Carvalho, Eric Wong, Nuno Moreno, Erwan Michard, Jorge Carneiro, Joaquín Rodríguez-Léon, Hen-Ming Wu, Alice Y. Cheung and José A. Feijó

*Plant Cell*; originally published online March 25, 2008;
DOI 10.1105/tpc.106.047423

This information is current as of May 2, 2016