Plasma membrane H\(^+\)-ATPases sustain pollen tube growth and fertilization

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Pollen tubes are highly polarized tip-growing cells that depend on cytosolic pH gradients for signaling and growth. Autoinhibited plasma membrane proton (H\(^+\)) ATPases (AHAs) have been proposed to energize pollen tube growth and underlie cell polarity, however, mechanistic evidence for this is lacking. Here we report that the combined loss of AHA6, AHA8, and AHA9 in Arabidopsis thaliana delays pollen germination and causes pollen tube growth defects, leading to drastically reduced fertility. Pollen tubes of aha mutants had reduced extracellular proton (H\(^+\)) and anion fluxes, reduced cytosolic pH, reduced tip-to-shank proton gradients, and defects in actin organization. Furthermore, mutant pollen tubes had less negative membrane potentials, substantiating a mechanistic role for AHAs in pollen tube growth through plasma membrane hyperpolarization. Our findings define AHAs as energy transducers that sustain the ionic circuit defining the spatial and temporal profiles of cytosolic pH, thereby controlling downstream pH-dependent mechanisms essential for pollen tube elongation, and thus plant fertility.
Flowing plants evolved a complex process to deliver sperm cells for double fertilization and seed formation. When pollen, the male gametophyte, lands on a receptive stigma of a flower, it germinates to produce a cell extension, the pollen tube, which carries non-motile sperm cells. Pollen tubes grow through the female sporophytic tissues and discharge the sperm cells into the female gametophyte, the embryo sac.

Pollen tubes elongate by tip growth, a mechanism of cell elongation in which secretory vesicles fuse with the plasma membrane exclusively at the growing apex, that is common to root hairs, filamentous fungi, and developing neurons. Pollen tubes evolved to be streamlined chemotropic growing cells. They possess dramatically polarized cytosolic ion gradients at the tip (H\(^+\), Ca\(^{2+}\), Cl\(^-\)), which are believed to modulate the cellular processes and structures involved in tip growth, including the actin cytoskeleton. Although cytosolic buffering mechanisms were thought to preclude the existence of cytosolic pH gradients, pollen tubes were shown to have apical gradients with up to one pH unit ranging over 10–30 µm from the extreme tip of the pollen tube. These gradients correlate spatially with a functional 

**Results and discussion**

**AHA6, AHA8, and AHA9 are plasma membrane proton ATPases.** The *A. thaliana* genome encodes 11 presumably functional H\(^+\)-ATPases (AHA1–11), of which the three isoforms AHA6, AHA8, and AHA9 from *Arabidopsis thaliana* are essential for pollen tube growth and fertility. Triple mutants of these pumps have strongly reduced fertility due to premature pollen tube arrest, and mutant pollen tubes showed reduced extracellular ion fluxes, reduced plasma membrane potential, disturbed spatiotemporal patterns of cytosolic pH and disrupted actin organization. We conclude that the AHA6s energize a loop of H\(^+\) current around the tip, which is essential for pollen tube function.

In the *aha6/8* double mutant, AHA6::GFP fully complemented the loss of AHA6 (Supplementary Fig. 4a). AHA8::GFP partially rescued the aberrant phenotype (whereas AHA8 without GFP fully rescued it). AHA9::GFP fully rescued the *aha6/9* phenotype of reduced seed setting (Supplementary Fig. 4a).

We confirmed the H\(^+\) pump activity of each of these proteins by heterologously expressing cDNA containing their coding region in the yeast strain RS-2, in which the native proton pump PMA1 is under the control of a galactose-inducible promoter. This strain can only grow in the absence of galactose when a functional plasma membrane H\(^+\) pump is expressed. As a C-terminal autoinhibitory domain has been identified in plasma membrane H\(^+\)-ATPases, we also expressed gene versions that encode truncated pumps lacking 92 C-terminal amino acid residues. The autoinhibitory C-terminal domain of AHA6 does not block the activity of the pump, but controls the degree of coupling between ATP hydrolysis and H\(^+\) pumping, i.e., the pumping efficiency. Thus, the ability of full-length AHA6s to complement *S. cerevisiae pma1* depends on the size of the electrochemical H\(^+\) gradient the pumps are pumping against, if growth is only reduced, the period of time of the growth assay. The truncated versions of all three pumps complemented *pma1* even at an external pH of 3.5, confirming a H\(^+\)-extrusion pump function (Fig. 1b). Expression of full-length cDNA did not complement growth, suggesting that the autoinhibitory domain strongly reduces proton pumping in the yeast system (Fig. 1b).

We then assayed the specific ATPase activity of the C-terminally truncated pumps as a function of pH. All three gametophyte-specific pumps had their maximum activities close to pH 6.5 (Fig. 1c). This is similar to the pH optimum of AHA2, an isoform strongly expressed in the vegetative parts of the plant (the sporophyte). Moreover, all pumps had overlapping *K_m* values for ATP (Fig. 1d), suggesting that sporophyte and gametophyte H\(^+\)-ATPases have similar pump properties.

**ahae6/8/9 pollen tubes have severe growth phenotypes.** We investigated the roles of AHA6, AHA8, and AHA9 genetically by characterizing T-DNA insertion lines in *A. thaliana* (Fig. 2a). The *ahae6-1*, *ahae8-1*, *ahae8-3*, *ahae9-4*, and *ahae9-5* mutant plants were confirmed to have undetectable levels of the corresponding RNA transcript in mature flowers of homozygous T-DNA insertion lines (Fig. 2b, c). Of the single mutant plants, only *ahae6* showed mild phenotypes (Supplementary Table 1; Supplementary Fig. 3a–c).

Given that genetic redundancy is a well-established phenomenon in ion transporter families of pollen, we crossed the individual lines to generate homozygous double and triple mutant plants. When self-fertilized, the three combinations of double mutants (*ahae6/8*, *ahae6/9*, and *ahae8/9*) showed different degrees of early pollen tube growth arrest (Fig. 2d, e), reduced pollen tube elongation rates (Fig. 2e), and reduced fertility (Fig. 2f). *ahae6/8* pollen tubes grew at a similar rate as those of the wild-type but terminated growth much earlier, whereas *ahae6/9* pollen tubes...
germinated later and grew slower than those of the wild-type but grew almost as long. This suggests that AHA6 and AHA9 together function in pollen tube germination and in the early phases of tube elongation, whereas AHA6 and AHA8 together are important for sustained pollen tube growth. All reported phenotypes could be functionally complemented by transforming the absent AHA isoforms under their native promoters back into the mutant lines (Supplementary Fig. 4). Male transmission of the fertility defects was confirmed by reciprocal crosses between the WT and plants bearing aha alleles (Supplementary Table 2).

Aiming to further reduce the effects of redundancy in AHA function, we generated aha6, aha8, and aha9 triple mutants (Table 1). Pollen maturity and viability were confirmed with DAPI and Alexander stain, respectively (Supplementary Fig. 5). Pollen tube growth in planta was strongly affected, with only a few pollen tubes succeeding to grow through the stigma into the ovary, resulting in a reduction to about 3–10 seeds per plant (Fig. 2d–f). Likewise, in vitro germination of the triple mutant pollen was significantly reduced (Fig. 2g), with erratic or wavy pollen tubes forming that were significantly shorter than those of the wild-type (Fig. 2i).

Although aha6/8/9 exhibited premature pollen tube growth arrest, initial growth still occurred but at a reduced rate (Fig. 2h, Supplementary Fig. 6). A likely explanation is that one or more additional AHA isoforms is involved in pollen tube growth during the early growth phases. AHA7 is also expressed in pollen tubes, but at a much lower level than AHA6, AHA8, and AHA9 (Supplementary Fig. 1). Loss of AHA7 led to a 30% reduction in seed set, but only when AHA6 and AHA9 were also lacking (Supplementary Fig. 7).

Even after genotyping >500 progeny of triple mutant plants that were pollinated with pollen from plants that were heterozygous for the fourth allele, aha6/7/8/9 quadruple mutants were not identifiable (Supplementary Fig. 7). This suggests that AHA7 functions redundantly with the other isoforms, and may weakly sustain pollen tube growth in the aha6/8/9 mutant.

Fig. 1 AHA6, AHA8, and AHA9 are proton pumps localized in the plasma membrane of pollen tubes. a Left, Histochemical staining for GUS activity in pollen grains and grown pollen tubes stably expressing AHA promoter::GUS fusions and observed using a light microscope. Right, Pollen tubes stably expressing GFP-tagged AHA proteins under control of their endogenous promoters and observed using a confocal microscope (see also Supplementary Fig. 2b). AHA6::GFP and AHA8::GFP were expressed in the aha6/8 mutant and AHA9::GFP was expressed in the aha9 mutant. Scale bars = 10 μm. b Mutated AHA6, AHA8, or AHA9 proteins lacking the autoinhibitory C-terminal domain, but not the full-length proteins, support yeast growth in the absence of its own plasma membrane proton ATPase, PMA1. The yeast strain R5-72 used in this analysis has PMA1 under control of a galactose-inducible promoter and does not express the gene on glucose medium. c Specific ATPase activity of recombinant full-length and C-terminally truncated AHA proteins assayed between pH 3.5 and pH 8.5. d Specific ATPase activity of recombinant full-length and C-terminally truncated AHA proteins assayed at pH 6.5 and with various ATP concentrations.
**Fig. 2** AHA double and triple mutants display aberrant pollen tube phenotypes.  

**a** Diagram of gene structures with position of T-DNA insertions (triangles) in the genes. Red arrowheads indicate the T-DNA left border. Exons are indicated as black rectangles, and introns as black lines. Primers used for reverse-transcription PCR (RT-PCR) are indicated by short black lines above the gene representations.  

**b** RT-PCR analysis of T-DNA insertion lines using primer pairs indicated in (a). RNA was from flowers at stage 1271. PP2AA3 served as a positive control.  

**c** RT-PCR analysis of T-DNA insertion lines using primers that amplify full-length cDNA of AHA8.  

**d** Aniline blue-stained pollen tubes of ms1 pistils pollinated with different aha mutant lines. White arrows indicate the position of the longest tubes at 48 h after pollination.  

**e** In vivo pollen tube growth rate (as a fraction of pollen tube length over pistil length), as revealed by aniline blue staining (n ≥ 3 for all genotypes and time points; error bars show SD).  

**f** Number of seeds per silique (one-way ANOVA and Bonferroni’s Multiple Comparison Test; p < 0.01; error bars show mean with SD).  

**g** In vitro germination rate for WT and aha6/8/9 triple mutant pollen. Means from three individual experiments with ≥ 100 scored pollen grains for each genotype and replicate are shown (two-sided t-test; p < 0.003).  

**h** Average pollen tube growth rate assayed in vitro (one-way ANOVA; Bonferroni’s test; p < 0.01; error bars show mean with SD).  

**i** Light microscopy images of WT and aha6/8/9 pollen tubes grown in vivo. The triple mutant is characterized by short and wavy pollen tubes. Scale bar = 100 µm.
Another possible candidate is AHA3, which is expressed in microspores27. However, this possibility could not be studied, since AHA3 deletion mutants failed to produce pollen28. Together, our data suggest that AHA6, AHA7, AHA8, and AHA9 are needed for proper pollen tube germination and elongation and that these pumps function redundantly in the pollen tube.

**Ion fluxes are reduced throughout aha6/8/9 pollen tubes.**

Having established the genetic basis of AHA function in pollen tube growth, we focused on its mechanistic basis in cell physiology. We used an extracellular ion-selective vibrating probe to measure H⁺ flux profiles along the pollen tube length, as these profiles can be taken as reporters of AHA activity17 (representative wild-type pollen tube in Fig. 3a). The growth rate reduction in all mutant combinations lacking AHA6 (Fig. 2c, h) was associated with reduced H⁺ influx at the tip (Fig. 3b), lower efflux at the shank, and a retraction of the influx/efflux reversal boundary towards the shank (Fig. 3c), with more dramatic effects in the triple mutant. Wild-type pollen tubes showed an inversion of net H⁺ influx at the tip to a net efflux at a distance of ca. 15–20 µm from the tip (Fig. 3c), while all mutant combinations lacking AHA6 have virtually no net efflux along the tube (Fig. 3c). Although aha8/9 shows an inversion point beyond 20 µm from the tip (Fig. 3c), the influx at the tip and efflux along the shank are comparable to those of the wild-type.

Large anionic fluxes were hypothesized to function in water movement, turgor, and elongation in growing pollen tubes29, with efflux at the apex and influx along the tube and a corresponding negative cytosolic concentration gradient at the tip30–32. Indeed, a striking reduction of anionic efflux was observed in the double and triple mutants lacking AHA6, while aha8/9 showed wild-type-like fluxes (Fig. 3d), in agreement with the growth phenotype observed in vivo and in vitro (Fig. 2e, h). Anionic efflux at the tip, which contributes to the largest electrical currents in growing lily (Lilium longiflorum) pollen tubes33, appears to respond to the electrochemical imbalance generated by AHAs at the shank, which would be expected to contribute to a net hyperpolarization of the plasma membrane32,34. Indeed, cationic influx at the tip can be elicited by hyperpolarizing pulses applied to the shank of lily pollen tubes35, supporting a link between tip fluxes and shank membrane potential.

A cytosolic gradient of H⁺ is reduced in aha6/8/9 tubes. Since mutants lacking AHA6 virtually lacked H⁺ efflux at the shank of the pollen tube, while retaining some, albeit a reduced, influx at the tip (Fig. 3b, c), we expected their pollen tubes to have a higher cytosolic H⁺ concentration and a smaller gradient between the tip and shank. We assessed cytosolic H⁺ concentrations by transforming all mutant lines with the ratiometric probe pHluorin, which was calibrated using the H⁺ ionophore nigericin and medium with pH 5.8–8.0 (Supplementary Fig. 8a). The pH gradients were estimated using a custom protocol36.

**A. thaliana** wild-type pollen tubes had a cytosolic [H⁺] gradient, with a higher pH in the shank than in the tip region, in agreement with the localization pattern of the H⁺-exporting AHA6, AHA8, and AHA9 pumps (Fig. 3a). Accordingly, a lower cytosolic pH was observed at the tip and shank in all mutant combinations lacking AHA6 than in the wild-type (Fig. 3e). In the apex, the pH of the aha6/8/9 pollen tubes was ca. 0.3 units lower than that of the wild-type (Fig. 3e, Supplementary Fig. 8b and exemplified in Fig. 4a). Even more pronounced differences were observed in the shank, where the pH of the mutant was ca. 0.5 units lower than that of the wild-type (Fig. 3e; Supplementary Fig. 8c; Fig. 4a). These changes in cytosolic pH translate into a shallower tip-to-shank pH gradient (Fig. 3f, detailed in Fig. 4a). The reduction in growth rate observed in AHA mutants is thus associated with the difference in pH and the gradient itself, suggesting a mechanistic relationship between growth and cytosolic pH. However, only the tip/shank gradient showed a significant correlation with average growth rate both across (Fig. 3g) and within the time series (Supplementary Fig. 9), while absolute pH at the tip did not (Supplementary Fig. 9).

Tip pH is considered to be involved in growth regulation, since H⁺ influx into the tip, but not the shank, can change the directionality of pollen tube growth as previously shown in tobacco. Application of the cation ionophore gramicidin A to the apical area, where NtAHA1 is absent, resulted in cytosol acidification concomitant with reorientation of pollen tube growth, but had no effect when applied to the shank, where NtAHA1 is present37. Similarly, growing lily pollen tubes subjected to an altered pH regime at the tip induced by water electrolysis on an active electrode either bent away from the electrode, stopped growing, or bursted36. Ion gradients have been implicated in multiple roles directly related to growth regulation, such as orienting actin polymerization and consequently cytoplasmic streaming, and vesicle/organellar dynamics38. Oriented vesicle movement has been hypothesized to occur spontaneously in pollen tubes by three basic mechanisms: (i) up a concentration gradient, (ii) towards the most depolarized plasma membrane domains, or (iii) along with osmotically driven fluid motion34. Changes in gradient steepness are expected to affect all such processes, thus possibly accounting for the reduction in average growth rate found in mutants lacking AHA6. These results indicate that AHAs are key regulators of the mean level and gradient of cytosolic H⁺ concentration.

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Table 1 Offspring of self-pollinated lines exhibit a non-Mendelian pattern of inheritance.

| Homozygous background (parent) | Heterozygous allele (parent) | AHA/AHA (progeny) | AHA/aha (progeny) | aha/aha (progeny) | Ratio | $\chi^2$ | P value $^b$ |
|---------------------------------|---------------------------|-------------------|-------------------|------------------|-------|---------|-----------|
| aha6-1                          | AHA8/aha8-1               | 46                | 38                | 2                | 1:0.8:0 | 46.19   | <0.0001 |
| aha6-1                          | AHA9/aha9-4               | 91                | 116               | 19               | 1:3:0.2 | 46.04   | <0.0001 |
| aha8-1                          | AHA6/aha6-1               | 65                | 60                | 5                | 1:9:0.1:0 | 56.15  | <0.0001 |
| aha6-1                          | AHA9/aha9-4               | 18                | 42                | 21               | 1:2:3:1 | 0.33    | 0.847    |
| aha8-1                          | AHA6/aha6-1               | 78                | 85                | 16               | 1:1:1:0:0 | 43.40  | <0.0001 |
| aha6-1                          | AHA8/aha8-1               | 18                | 50                | 19               | 1:2:8:1:1 | 0.374  | 92.01   | <0.0001 |
| aha6-1                          | AHA9/aha9-4               | 175               | 209               | 36               | 1:1:2:0:2 | 0.577  | <0.0001 |
| aha6-1                          | AHA8/aha8-1               | 46                | 39                | 0                | 1:1:0:0 | 44.71   | <0.0001 |
| aha6-1                          | AHA9/aha9-4               | 41                | 35                | 0                | 1:0.9:0:0 | 44.71  | <0.0001 |

*Alleles were detected in T1 seeds by PCR. The expected ratio for non-compromised gametes is 1:2:1.

$^b$Bold font highlights significant differences.
Mutant tubes have a lower frequency of H\(^+\) oscillations. When cytosolic pH was quantified as a function of time using CHUKNORIS\(^{37}\), we observed pronounced pH oscillations in all genotypes (Fig. 4b, c, Supplementary Fig. 10). The aha6/8/9 triple mutant showed the largest difference from the wild-type, with an increased period of synchronized oscillations between growth and H\(^+\) cytosolic concentration \([H^+]_{cyt}\) (Fig. 4d). The triple mutant is also more irregular in terms of variability in oscillation period between different pollen tubes (Supplementary Fig. 11a, b), changes in phase relationship (Fig. 4e), and highly variable delays between growth and \([H^+]_{cyt}\) (Supplementary Fig. 11c).
and as a meshwork of very short, highly dynamic thin microfilaments in the apical dome\textsuperscript{14,45}. The sub-apical structures are fundamental for tip growth. Actin bundles along the shank end at this location where they are reorganized to sustain the inverted-fountain streaming pattern, with cortical motion towards the tip, and central motion towards the grain. The molecular mechanisms underlying this actin remodeling mechanism and, fundamentally, its spatial definition, remain poorly understood. Yet, pH is an established regulator of the ADF/cofilin family of actin remodeling proteins\textsuperscript{46–48}, which drives the turnover and depolymerization of entangled F-actin\textsuperscript{49} and has been shown to function in actin organization in pollen tubes\textsuperscript{44,50}.

We thus explored the role of pH in regulating actin turnover in pollen tubes, by staining actin with Alexa 488 phalloidin\textsuperscript{51,52}. In addition to lacking a sub-apical actin fringe, the volume of the apical 5 µm of \textit{aha6/8/9} mutant pollen tubes was devoid of actin (Fig. 5b). Given the alterations in cytosolic pH and the tip-focused H\textsuperscript{+} gradient in the absence of AHAs, the changes in actin distribution are likely caused by differences in the threshold of this tip-focused gradient. This interpretation is further in accordance with the cytosol acidification-induced disruption of actin filament organization in lily pollen tubes\textsuperscript{44}.

On the other hand, cytosol alkalization promoted by plasma membrane H\textsuperscript{+}-ATPase was proposed to stimulate ADF, leading to depolymerization of F-actin and more plus ends, which then stimulate actin filament polymerization and, eventually, growth\textsuperscript{44}. Interestingly, \textit{aha6/8/9} phenocopies mutants in actin turnover factors that also display disrupted apical actin fringes and wavy growth, such as \textit{adf10} (normally present throughout the tube)\textsuperscript{53}, \textit{CAP1} (which acts in synergy with the ADF/cofilin family)\textsuperscript{54–56}, and \textit{A. thaliana} formin homology3 (AtFH3) and AtFH5\textsuperscript{57}. A shallower pH gradient (Fig. 3e, f) could also impact the orientation of actin fibers, since a gradient in pH should result in a gradient of ADF activity, which is pH dependent\textsuperscript{46–48} and hence could spatially define domains of re-organization and de novo polymerization.

Together, these results suggest that the abnormal growth and erratic shape (Fig. 2i) of AHA mutant pollen tubes are caused by a defect in actin turnover at the pollen tube tip (Fig. 5b), induced by reductions in cytosolic pH, pH gradient, and frequency of H\textsuperscript{+} oscillations (Fig. 4 and Supplementary Fig. 11).

Membrane potentials are less hyperpolarized in \textit{aha} mutants. AHAs are electrogenic pumps that establish a pH gradient across the membrane by pumping H\textsuperscript{+} out of the cell, thus hyperpolarizing the cell to more negative values of membrane potential. For quantitative comparisons of the membrane potentials in wild-type and mutant pollen tubes, we took advantage of ANNINE-6 plus (Fig. 5d), which is a voltage-sensitive fast-response dye that has been used for sensitive optical recording of neuronal excitation\textsuperscript{58}. Although methodological proof-of-principle demonstrations have been published\textsuperscript{59,60}, this dye has not been used for

**Fig. 3 Reduced pollen tube growth in \textit{aha6} double and triple mutants is associated with reduced extracellular ion fluxes and intracellular pH gradients.**

\textbf{a} Representative WT pollen tube summarizing H\textsuperscript{+} fluxes measured at the surface (arrows) and cytosolic pH gradient (false color). Arrow size is scaled with the flux intensity shown on the bottom bar, while direction denotes influx or efflux. \textbf{b} Extracellular H\textsuperscript{+} fluxes at the pollen tube tip. Violin plots show the probability density with color-filled curves obtained from individual observations (open gray circles), with boxplots (thick black lines and outliers as black dots) overlaid with the mean and standard error (red circle and lines). \textbf{c} Extracellular H\textsuperscript{+} fluxes throughout the pollen tube sampled every 5 µm, averaged and interpolated with a local polynomial fit (loess) with n > 10 for all genotypes. Negative values indicate influx and positive values efflux. \textbf{d} Extracellular anion efflux at the tip. \textbf{e} Cytosolic pH throughout the tube averaged with loess for each genotype (n > 16), obtained by fluorescence imaging of a calibrated pH probe (pHluorin). Media pH indicated by dashed line. \textbf{f} Gradient steepness estimated by the fold change in cytoplasmic pH between the tip and shank (subcellular regions defined as in Fig. 4). \textbf{g} Average growth rate correlates with pH ratio between the tip and shank (linear fit in blue with standard deviation shaded), while its does not correlate with tip pH alone (Fig. 59). Where applicable, asterisks indicate a significant difference (p < 0.01) compared to the control (WT); one-way ANOVA followed by the post-hoc Dunnett test.

All genotypes showed complex synchronized oscillations between [H\textsuperscript{+}]\textsubscript{cyt} and growth rate, with multiple frequencies co-existing in the same sequence, but with drastic changes in period over time (Supplementary Fig. 10), although with no overall difference in amplitude (Supplementary Fig. 11d). Slower oscillations could contribute to the drastically premature growth arrest of AHA6 mutants (Fig. 2d, e), presumably through critical effects on time-sensitive processes such as actin polymerization and cell wall rigidity\textsuperscript{7}. AHAs could affect tip oscillations through the electrochemical imbalance generated in the shank plasma membrane, leading to hyperpolarization in the shank and a more depolarized tip\textsuperscript{52,34}, or via pH/voltage sensitive channels localized throughout the pollen tube, such as the recently characterized H\textsuperscript{+}/Cl\textsuperscript{−} co-transporter TMEM1638\textsuperscript{38}.

**Turgor pressure is not altered in mutant pollen tubes.** After having identified spatio-temporal changes in intracellular pH and extracellular ion fluxes in AHA mutants, we investigated its possible effect on the mechanism underlying the observed defect in pollen tube growth. The plasma membrane H\textsuperscript{+}-ATPase has been proposed to be the powerhouse driving pollen tube growth\textsuperscript{34,14}. Plasma membrane H\textsuperscript{+}-ATPases energize the plasma membrane and drive the uptake of solutes through H\textsuperscript{+}-coupled co-transporters and channel proteins, followed by the uptake of water, which results in an increase in turgor pressure. The reduced ion fluxes in the mutant pollen tubes could thus lead to a decrease in turgor pressure, which could underlie the observed elongation defects. To test this hypothesis, we estimated turgor in growing pollen tubes using the incipient plasmolysis technique\textsuperscript{39}. Despite severe tube growth phenotypes in \textit{aha6/8/9} (Fig. 2e, i), turgor pressure in wild-type and mutant pollen tubes was not significantly different, with an average of ~0.2 MPa (Fig. 5a), which is in agreement with the results of previous studies\textsuperscript{38,39}.

The lack of correlation between internal turgor pressure and growth rate of pollen tubes\textsuperscript{39} (reviewed in refs. \textsuperscript{40,41}) contradicts the assumption that turgor is modulated during oscillatory growth\textsuperscript{9,45}. Our results support a tip growth model, which is not dependent on increased turgor pressure, and suggest that the combined fluxes of the opposing circuits of cations and anions at the tip and shank balance each other out, so that there is no net build-up of osmotically relevant ionic concentrations\textsuperscript{34}.

**Actin cytoskeleton organization at the tip is compromised.** We then investigated the actin cytoskeleton as a possible target of the pH alterations that could explain the aberrant growth of \textit{aha6/8/9} pollen tubes (Fig. 2i). Actin is essential for pollen tube growth and is organized in bundles along the shank of the tube, ending in a basket- or cortical fringe-like structure in the sub-apical region (~12–20 µm from the tip in lily, likely ~3–6 µm in Arabidopsis) and as a meshwork of very short, highly dynamic thin
plant cell phenotyping. We focused on the sub-apical zone (10–20 µm distal from the tip, where AHA labeling fades away) and the AHA-stabilized shank (40–50 µm from the tip) (Supplementary Fig.12 a–e). From this analysis, it was evident that the aha6/8/9 mutants had less hyperpolarized membrane potentials than the wild-type, (<50% fluorescence) (Fig. 6d). The wild-type membrane potential has previously been determined to be at −127 mV61. As the linearity of the ANNINE6-plus response at such negative values has not been determined, one cannot extrapolate exact values for the mutant, but given the linearity of the dye response over 120 mV (reviewed in ref. 58), values more positive than −70 mV should be expected for the triple mutant.
We further confirmed this result using the oxonol dye DiBAC$_4$(3), a slow-response voltage-sensitive dye that enters depolarized cells and is excluded from hyperpolarized cells. We have assayed DiBAC$_4$(3) under normal conditions and after wortmannin treatment to prevent possible endocytosis artifacts on dye uptake. Under all conditions, the ah$a$6/8/9 triple mutant had an increased degree of DiBAC$_4$(3) uptake compared to the WT, which is indicative of a less negative potential (Supplementary Fig. 12f).

The quantitative accuracy of ANNINE-6 and the confirmation of its main conclusion using DiBAC$_4$(3) provide compelling evidence that pollen tubes of AHA mutants, which are impaired in growth and fertility, have less hyperpolarized membrane potentials, thus substantiating a mechanistic role for AHAs in pollen tube growth through plasma membrane energization.

This work provides the first genetic evidence that plasma membrane H$^+$-ATPases energize the plasma membrane and control dynamic H$^+$ fluxes in growing pollen tubes and are required for apical elongation of pollen tubes and hence successful fertilization. Furthermore, these proton pumps underlie the spatiotemporal control of the pH homeostasis set point, establishing a more acidic apex than shank region and a pattern of proton oscillations that is synchronized with pollen tube growth. We propose that the spatial distribution of plasma membrane H$^+$-ATPases, which are present only at the tube shank, mediates the formation of a [H$^+$]$_{cyst}$ gradient that directs actin bundling near the growing tip, and generates a proton motive force that drives the anion efflux and H$^+$ influx at the tip, producing an oscillatory ion circuit that coordinates pollen tube growth (Fig. 6).

Local ionic fluxes precede pattern formation and polarized growth in a diverse array of tip-growing cells such as algal zygotes, fungal hyphae, and growing neurons (reviewed in ref. 33), such that depolarization of the tip may be a pre-condition to sense external stimuli. Indeed, ion gradients have been reported in virtually all biological models for polar growth, while H$^+$ has long been proposed as a crucial signal for pollen tube growth in particular. Our results are in line with such models, supporting the notion that AHAs have multiple effects on tip growth. Furthermore, the approach presented here might help elucidate the mechanisms driving growth in other types of tip-growing cells.

**Methods**

**Plant materials and growth conditions.** Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type plant. T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were sown on soil and vernalized in darkness at 4 °C for 2 days before being transferred to climate chambers maintained at 22 °C with 70% humidity and a 16-h-light/8-h-dark cycle (100 μmol, m$^{-2}$s$^{-1}$). For ion flux measurements, plants were grown at 22 °C with 70% humidity and a 12 h light/12 h dark cycle (100 μmol m$^{-2}$ s$^{-1}$).

**Characterization of T-DNA insertion mutants.** Genomic DNA was used to identify heterozygous and homozygous lines by PCR. Gene-specific primers together with specific T-DNA left border primers (Supplementary Table 3) were used to genotype T-DNA insertion lines. All T-DNA insertion lines were verified by sequencing. AHA9 transcript levels in Col-0 and the respective mutants. Total RNA was extracted from flowers (10 flowers per sample; at flower stage 12f) using a Plant RNeasy Kit (Qiagen) according to the manufacturer’s instructions. To synthesize cDNA, 1 mg RNA was reverse transcribed using the Script Select cDNA Synthesis Kit (BioRad), according to the manufacturer’s instructions. RT-PCR was performed using 5 ng cDNA (or 1 ng gDNA extracted from a Col-0 plant) as template and cDNA-specific primer pairs (Supplementary Table 3) were used to determine the levels of full-length and partial transcripts. PPA2A3 (At1g13320) was amplified as a positive control for RT-PCR analysis (Supplementary Table 3).

The ah$a$9-1 allele had a pleiotropic phenotype for heterozygous, but not homozygous, plants, probably due to chromosomal rearrangements, and was thus not further characterized.

**Segregation analysis.** Plants heterozygous for a T-DNA insertion allele were allowed to self-fertilize and the resulting seeds were sown on soil in 96-cell plug trays. Genomic DNA was extracted from leaves as described below and the alleles in question were amplified by PCR using sets of optimized primer pairs (Supplementary Table 3). For reciprocal crosses, unopened flowers of plants heterozygous for a T-DNA insertion were emasculated and stigmas were hand pollinated with WT pollen. Flowers of ms1 plants were pollinated with plants heterozygous for a T-DNA insertion. Developed seeds were collected from these siliques and sown on soil in 96-cell plug trays, and the genotypes were determined as described above.

**DNA extraction.** DNA was extracted in 96-well format. Leaves of young plants were collected in tubes each containing a 3 mm steel ball (Dansk Kuglecenter). Samples were frozen in liquid nitrogen and homogenized using a Retsch Tissue Lyser (Qiagen). Then, 280 μl Edward’s solution (200 mM Tris-HCl [pH 7.5], 250 mM NaCl, 50 mM EDTA, and 0.5% [w/v] sodium dodecyl sulfate) was added to each sample and the 96-well plates were centrifuged for 15 min at 9500 rcf at room temperature. Then, 125 μl buffer was transferred to fresh 96-well plates containing 125 μl 2-propanol and mixed by pipetting. Plates were centrifuged for 15 min at 9500 rcf. The supernatant was pipetted off and discarded, 300 μl 70% (v/v) ethanol was added to the pellet without resuspending it, and the samples were centrifuged for 5 min at 5900 rcf and room temperature to wash the pellets. The ethanol was removed and the samples were left to dry overnight at room temperature. The DNA was resuspended in 300 μl deionized water and the samples were maintained at room temperature for at least 3 h. DNA samples were diluted 10-fold in deionized water and for genotyping purposes 5 μl DNA was used per 20-μl PCR reaction.

**Construction of the promoter::gfp expression fusions.** To construct the pAHA6::AHA6::GFP, pAHA8::AHA8::GFP, and pAHA9::AHA9::GFP expression plasmids, genomic A. thaliana Col-0 DNA was amplified by Phusion High-Fidelity DNA-polymerase (NEB) using primer pairs that included the region 1747 (AHA6), 673 (AHA8), or 762 (AHA9) bp upstream of the start codon and the entire gene except for the stop codon (Supplementary Table 3). The resulting PCR products were cloned into pENTR/D-TOPO vectors (Invitrogen) by TOPO cloning, according to the manufacturer’s instructions, and the inserts were fully sequenced. Using LR recombinase (Invitrogen), the inserts were subsequently transferred into the pMD107 destination vector, such that the C-terminus of the inserts were fused in-frame to EGFP. The resulting expression vectors were then introduced into Col-0 and the ah$a$6/8 and ah$a$6/9 double mutant lines.

**Construction of the pAHa8::AHA8 construct.** To construct the pAHa8::AHA8 expression plasmid, genomic A. thaliana Col-0 DNA was amplified by Phusion High-Fidelity DNA-Polymerase (NEB) using the same forward primer as used for cloning the GFP construct, and reverse primer including the stop codon (Supplementary Table 3). The resulting PCR product was cloned into pENTR/D-TOPO vectors (Invitrogen) by TOPO cloning, according to the manufacturer’s instructions, and the inserts were fully sequenced. Using LR recombinase (Invitrogen), the
The resulting expression vector was then introduced into the aha6/8 double mutant line.

**Construction of the promoter∷GUS constructs.** To construct the pAHA6∷GUS, pAHA8∷GUS, and pAHA9∷GUS expression plasmids, genomic *A. thaliana* Col-0 DNA was amplified by Phusion high-fidelity DNA-polymerase (NEB) using the same forward primer as used for cloning the GFP constructs, and reverse primers complementary to sequences in the second exon of the respective gene (Supplementary Table 3). The resulting PCR products were cloned into pENTR/D-TOPO vectors (Invitrogen) by TOPO cloning, according to the manufacturer’s instructions, and the inserts were fully sequenced. Using LR recombinase (Invitrogen), the inserts were subsequently transferred into the pMDC162 destination vector.70.

![Fig. 5 Turgor pressure is unaltered in aha6/8/9 pollen tubes, but actin is located away from the tip and membrane potential is reduced.](image)

**Fig. 5 Turgor pressure is unaltered in aha6/8/9 pollen tubes, but actin is located away from the tip and membrane potential is reduced.** **a** Turgor pressure in WT and AHA mutant pollen tubes (one-way ANOVA and Bonferroni’s Multiple Comparison Test; *p* < 0.01; error bars show mean with SD). **b** Actin in pollen tubes was stained with Alexa 488 phalloidin and images were obtained using a confocal laser scanning microscope. Fluorescence and bright-field images were generated by maximum intensity projections of all optical sections for each pollen tube using ImageJ software (scale bars = 5 μm). **c** The distance from the tip of the pollen tube to the actin filaments in pollen tubes. Where applicable, asterisks indicate a significant difference compared to the control (WT); ANOVA with Bonferroni’s test; *p* < 0.01; error bars show mean with SD. **d** Quantification of plasma membrane potential using ANNINE-6-plus 10–20 μm (left panel) and 40–50 μm (right panel) from the tip, respectively. Violin plots show the probability density with color-filled curves obtained from individual observations (open gray circles), with boxplots (thick black lines and outliers as black dots) overlaid with mean and standard error (red circle and lines). Membrane fluorescence values were quantified using a customized protocol, including near super-resolution, Nyquist oversampling and normalization between replicates (Suppl. Fig. 12). Differences were significantly lower in all mutants than in wild-type, but no significant differences were found within mutants at either domain of the plasma membrane sampled (see Suppl. Fig. 12 for details) (scale bars = 2 μm).
shallower cytosolic pH gradient (color black arrow) vanish, leading to a lower pH throughout the tube with a for actin organization during pollen tube growth.

ligated using the T4 ligase (NEB), transformed into Shrimp Alkaline Phosphatase (rSAP, NEB). Vector and hybridized primers were (excitation, 546/10 nm; emission, 600/40 nm). The spatio-temporal control of ion fluxes and intracellular gradients required for actin organization during pollen tube growth. Therefore, the pLat52::pHluorin construct, the pLat52::pHluorin plasmid and pMDC99 were mixed and digested using Xhol and HindIII (NEB), effectively excising pLat52::pHluorin and the Gateway Cassette. The digestion product was ligated with T4 DNA ligase (NEB) and transformed into Escherichia coli strain TOP10. Positive clones containing the resulting pMP5249 vector were selected on LB plates supplemented with 50 µg ml⁻¹ kanamycin (Supplementary Fig. 13 for vector map).

Construction of the pLat52::pHluorin constructs. Ratiometric pHluorin is a pH-sensitive GFP variant71,72, which carries a cryptic intron72, leading to aberrant mRNA processing and the absence of protein expression. Silent mutations were introduced into the pHluorin coding sequence, using a primer pair (Supplementary Table 3), to eliminate the cryptic intron sequence and restore pHluorin expression during GUS activity. Samples were vacuum infiltrated for 5 min with 50 µl of chloro-3-indolyl-β-D-glucuronic acid (X-glca; Duchefa) solution (3 mM K$_2$(Fe(CN)$_6$), 3 mM K$_4$(Fe(CN)$_6$), 0.4% [w/v] Tween-20, 50 mM KH$_2$PO$_4$, pH 7.2, 2 mM X-glca) and incubated in the solution at 37 °C in darkness for 6 h (influorescences). Pollen were incubated for 3 h at 22 °C before staining for GUS activity at 22 °C for 20 h. After incubation, influorescences were cleared in 70% ethanol washed and air dried. Stained samples were observed with a Leica DMR HC light microscope (pollen) and a Canon ELPH110HS camera (influorescences).

Subcellular localization studies. The subcellular localization of AHA6, AHA8, and AHA9 tagged with EGFp in mature pollen and pollen tubes from the A. thaliana complementation lines was determined by confocal microscopy. Images were obtained with a Leica SP5, using a 40× oil immersion objective (NA 1.0; Leica). EGFp was excited with the 488-nm line of an argon laser and emission recorded from 495 to 550 nm. Optical sections were captured, and images were prepared by generating max-intensity projections (using ImageJ) of the optical sections through an individual pollen grain or pollen tube. Autofluorescence (monitored in wild-type mature pollen and pollen tubes) was negligible relative to EGFp fluorescence.

Full-length and C-terminally truncated cDNA constructs. Total RNA was extracted from A. thaliana Col-0 seedlings using an RNasey Plant Mini Kit (Qiagen) and RNA was reverse transcribed using the Script cDNA Synthesis Kit (BioRad) according to the manufacturers’ instructions. The PCR products of full-length coding sequences and C-terminally truncated sequences (see Supplementary Table 3 for primer sequences) were cloned into the pENTR/D-TOPO vectors (Invitrogen) by TOPO cloning, according to the manufacturer’s instructions, and the inserts were fully sequenced. Using LR recombinase (Invitrogen), the inserts were subsequently transferred into the destination vector pMP4409. pMP4409 was generated by cutting pMP625 containing the promoter and terminator of PMA1 (yeast endogenous plasma membrane H⁻/ATPase79) with XhoI and SpeI, removing AHA2, and inserting the gene into Gateway Cassette A (Invitrogen); the correct orientation of the Gateway Cassette A was verified by sequencing.

Yeast complementation assays. AHA6, AHA8, and AHA9 expression clones were transformed into Saccharomyces cerevisiae strain RS-72 (Mat a; ade1-100 his4-519 leu2-3; is112 pPMAI-pGAL1) to be used for complementation tests80. In RS-72 the natural constitutive promoter of the endogenous yeast plasma membrane H⁻/ATPase PMA1 is replaced by the galactose-dependent promoter of GALI. Since PMA1 is essential for yeast growth, RS-72 grows only on galactose medium. Using this strain, plasmid-borne plant H⁻/ATPases brought under control of the constitutive PMA1 promoter can be tested for their ability to rescue pma1 mutants on glucose medium. Yeast was grown for 3 days at 30 °C in liquid medium containing 2% galactose. Each complementation experiment was replicated independently three times. In drop tests, cells were diluted in sterile water and 5 µl was spotted on selective media. Growth was recorded after incubation for 3 days at 30 °C.

Selection of transformed plants. Seeds of infiltrated plants were surface sterilized by washing in 70% (v/v) ethanol for 10 min, bleach solution with 0.05% (v/v) Tween-20 for 5 min, and then rinsing three times in milliQ water. Seeds were plated on ½ strength Murashige-Skoog (MS) medium78 supplemented with 1% (w/v) sucrose and 50 mg ml⁻¹ hygromycin B. The plates were stored at 4 °C for 48 h in darkness, and then transferred to growth chambers at 20 °C with a 16 h light period. After 2 weeks, seedlings showing resistance to hygromycin B were transferred to soil and tested by PCR for the presence of the correct insert.

Isolation of pollen. Unless stated otherwise, pollen grains were isolated in the morning from newly opened flowers. For this, flowers occupying a volume of about 500 µl were collected in a microcentrifuge tube and 700 µl pollen germination medium composed of 5 mM CaCl$_2$, 5 mM MgCl$_2$, 15% [w/v] sucrose, pH 7.5) was added to each tube. The samples were vortexed for 2 min, and the pollen pelleted by centrifugation at 11,200 g for 2 min. Flowers and medium were removed and pollen were resuspended in fresh pollen germination medium.

GUS staining and analysis. To determine the tissue-specific expression of AHA6, AHA8, and AHA9, plants for each GUS-expressing line were selected and influorescences were stained for GUS activity. A. thaliana plants were transformed with pKM1.1 R vector (Supplemental Table 3), to eliminate the cryptic intron sequence and restore pHluorin expression. Yeast complementation assays were performed using a Leica DMR HC light microscope (pollen) and a Canon ELPH110HS camera (influorescences).
Protein expression, purification, and characterization. Microsomes harboring recombinant plasma membrane H^+ -ATPase were isolated from transformed yeast cells33. Assaying the transformed yeast cells harboring the tra+ phenotype, the ATPase was transformed into the RS-72 strain and transformed yeast strains were grown on minimal media w/o leucine. Positive clones were grown for two days in minimal medium with 2% galactose. Cells were transferred to YPD media for 20 h and harvested by centrifugation at 4,000g for 10 min. The cell pellets were washed with cold MillQ water twice and resuspended in a buffer containing 200mM glyc, 50 mM MES/KOH (pH 6.5), 10 mM EDTA, 1 mM dithiothreitol (DTT) and 0.4mM phenylmethylsulfonyl fluoride. Cells were lysed by vortexing with glass beads (0.5 mm in diameter) 5 times 1 min. The cell debris was removed by centrifugation at 14,000g for 15 min and the supernatant was centrifuged again at 100,000g for 1h to pellet the total membranes. The membrane pellet was washed with a buffer containing 20% glycerol, 50 mM MES/KOH (pH 6.5), 1 mM EDTA, 1 mM DTT and 0.4mM phenylmethylsulfonyl fluoride, centrifuged again and homogenized in the same buffer. The protein concentration was determined using the Bradford reagent and bovine serum albumin as standard. The resuspended membrane fractions were either assayed directly or flash frozen in liquid nitrogen and stored at -80°C until use. The ATPase activity was determined by assaying the liberation of phosphate according to the Baginski assay82 in microtiter plates.

Light microscope with a ×5 objective and filter BP 340/380, 435/48 nm (DAPI) and 523/36 nm (FITC). To assess cytosolic pH in pollen tubes, ratiometric pHluorin was calibrated using 140 mM KCl, 30 µM nigericin (Tocris Biosciences), and 10 mM sodium phosphate with molar ratios of mono- and dibasic forms appropriate for the given pH (5.8–8.0). Ratiometric images of wild-type and mutant pollen tubes were obtained with the same acquisition protocol, and the calibration curve fitted with linear regression was applied to estimate the intracellular pH in all lines analyzed. Kymographs were generated from time-lapse images using ImageJ (Multiple Kymograph plugin) averaging over a 7-pixel neighborhood along a manual trace through the pollen tube midline for both DAPI and FITC channels.

Time series and gradient analysis. The CHUKNORRIS algorithm was used to analyze and extract oscillatory parameters from ratiometric kymographs36,37. CHUKNORRIS detects the pollen tube tip with subpixel resolution in each time point of the kymographs by fitting a linear model to the fluorescence decay of the ratiometric fluorescence signal towards the background. Tip detection allows aligning kymographs by the apex and extracting growth and fluorescence time series throughout the tube. Each time series is detrended and smoothed using a discrete wavelet transform, which separates changes in baseline (trend) and noise from the periodic signal that is then further analyzed. Wavelet transforms are used to provide time-explicit estimates of period and amplitude for individual series, additionally with phase relationships and delays for pairs. Synchronization oscillations between tip pH and growth rate oscillations were compared across genotypes using averages of the significant periodic components detected with a cross-wavelet transform for each pair of series. Although periods are often distributed in multiples modes, extracting only the main mode yielded similar results. Gradients throughout the pollen tube were analyzed by averaging a tip-aligned kymograph with a local polynomial fit (loess) across all time points within the same growth regime (protocol detailed in ref. 34).

Turgor pressure measurement. The turgor pressure of pollen tubes was measured using the incipient plasmolysis method35. Pollen tubes were germinated on solid liquid pollen germination medium in glass bottom dishes. After the germination period, 60 µl of growth medium was collected for osmolality measurement (denoting ne). Pollen tubes were observed under an inverted bright-field microscope (Leica inverted DMI4000B), and one growing pollen tube was selected for analysis. Liquid pollen germination medium with 10% (w/v) sucrose and 10% (w/v) NaCl was added to the growth medium until the plasma membrane and the cell wall of the tube tip (nt). An aliquot of 60 µl of the solution was collected for osmolality measurement denoting ni. The osmolality was measured using an osmometer (Gonotec). Turgor pressure is given by ni – ne and the measured values in osmol·kg^-1 were converted to MPa at 25°C.

Alexa 488 phalloidin actin staining. Pollen tubes were germinated and grown on solid pollen growth medium for 5 h at 23°C. For actin staining38,39, the pollen tubes were fixed in fixation buffer (300 µM 3-maleimidobenzoic acid N-hydroxysuccinimide ester in liquid pollen germination medium) for 1 h. After fixation, pollen tubes were washed three times in wash buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 14% [w/v] sucrose, 0.05% [w/v] Nonidet P-40) for 10 min and rinsed with 200 mM Alexa Fluor 488 phalloidin in wash buffer supplemented with 1% (w/v) bovine serum albumen (BSA) overnight in darkness at 4°C. The stained pollen tubes were washed twice for 10 min with wash buffer and actin was observed using a confocal laser scanning microscope (Leica; TCS SP5) equipped with a x100 oil objective (1.4-numerical aperture HC PLAN).

Actin imaging and analysis. The images of actin in pollen tubes were captured using a Leica TCS SP5 confocal laser scanning microscope with a x100 oil objective (1.4-numerical aperture HC PLAN). Fluorescence of Alexa 488 phalloidin was excited with a 488-nm argon laser and the emission wavelength was set at 500–545 nm. Pollen tube images were generated for analysis by scanning all optical sections at a z-step size of 0.5 µm and analyzing using the LAS AF Lite software (Leica; Version 2.6.0). To measure the distance from the plasma membrane to the sub-apical actin fringe, five individual lines were drawn parallel to the pollen tube, from the plasma membrane at the tip to the end of the actin filament structure. The length

Ratiometric [H^+] imaging and pHluorin calibration. Transgenic wild-type pollen tubes expressing the modified ratiometric pHluorin driven by the LAT3 promoter39 were imaged every 4 s on an Applied Precision Deltavision Core system, mounted on an Olympus inverted microscope, equipped with a front-illuminated sCMOS camera (2560 x 2160, pixel size 6.45 µm), and an InsightSHI fluorescence illuminator, using a ×63 1.2NA water immersion objective. Filter sets were set for excitation 490/18 nm (DAPI) and 475/28 (FITC). Emission filter sets were: 435/48 nm (DAP) and 523/36 nm (FITC).
is expressed as the average value calculated for the distance from the plasma membrane to the point of maximum fluorescence intensity on each of the five lines.

**Measurement of plasma membrane potential.** For Aninna-6 plus membrane potential measurements, wild-type and mutant pollen grains were collected and germinated as described above. Pollen grains were incubated at 21.5 °C for at least 2 h before adding the membrane potential dye Aninna-6 plus to a final concentration of 10 µM. Fluorescence images were acquired on a Zeiss LSM 980 Airyscan 2 Laser Scanning Confocal using a 63x NA1.4 oil immersion objective, in the Super-Resolution mode, and were processed with Airy Scan Super Resolution processing software in Zen Lite. The estimated resolution was better than 0.2 µm. Filter settings were as follows: excitation, 475 nm and emission, 499–549 nm (dual-band BP) and 573–627 nm (BP). Images were analyzed in Imagej by drawing transects that were 2 µm apart and perpendicular to the pollen tube growth axis (see Supplementary Fig. 12). Membrane fluorescence values were averaged from the three maxima pixels in each transect for both the left and right sides of the shank (Supplementary Fig. 12c), corresponding to better than a tenth of the three maxima pixels in each transect for both the left and right sides of the shank (Supplementary Fig. 12c), corresponding to better than a tenth of the three maxima pixels in each transect for both the left and right sides of the shank (Supplementary Fig. 12c), corresponding to better than a tenth of the three maxima pixels in each transect for both the left and right sides of the shank.

**Accession numbers.** Sequence data from this article can be found in GenBank/EMBL or the Arabidopsis Genome Initiative database under the following accession numbers: AHA6 (AT2G07560), AHA7 (AT3G60330), AHA8 (AT3G24640), and AHA9 (AT1G86600). The following T-DNA insertion lines were used in this study: SAIL_1293_D09 (aha1-6), SALK_042485 (aha7-1), SALK_105742C (aha7-2), SAIL_823_E09 (aha8-1), SALK_208753 (aha8-3), SALK_064821 (aha9-1); showed aborted male and female gametophytes is heterozygous lines; probably having chromosomal rearrangement and was not further analyzed) SALK_127945 (aha9-4), and GK-764H12 (aha9-5).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All data supporting the findings of this study are included in this published article, its Supplementary Information, and the Source Data file. Any other data and material will be made available upon reasonable request.

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2. Johnson, M. A., Harper, J. F. & Palanivelu, R. A fruitful journey: pollen tube ion fluxes between 10 µm and 40–50 µm from the tip (D. E., for analysis using DiBAC4(3) pollen tubes of wild-type and aha6/8/9 plants were germinated and grown for 3 h and thereafter stained with 2.5 µM DiBAC4(3) for 10 min before observation. Controls for endocytosis of the dye were made by treating the pollen tubes with 0.8 µM wortmannin in the liquid pollen germination medium for 1 h after pollen growth and staining with FM4-64 (2 µM) for 10 min immediately before observation. FM4-64 and DiBAC4(3) were excited with a 515- and 488-nm argon laser, respectively. The emission wavelength of FM4-64 was set at 620–660 nm, and that of DiBAC4(3) at 515–565 nm using a confocal microscope (Leica SP5). DiBAC4(3) fluorescence was measured as the intensity in the cytosol area in the pollen tube tip and shank (40–50 µm from the tip) using ImageJ software (https://imagej.nih.gov/ij/).

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**Author contributions**

R.H., M.T.P., L.O., D.D., M.H., J.F., and M.P. designed the research; R.H., M.T.P., L.O., D.D., M.H., C.N., J.P., P.L., and C.C. performed the research. R.H., M.T.P., L.O., D.D., M.H., C.N., J.P., P.L., J.F., and M.P. analyzed the data. R.H., M.T.P., L.O., D.D., M.H., J.F., and M.P. wrote the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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