

Effects of extracellular NH_4^+ , NO_3^- , and H_2PO_4^- on root hair elongation in *Arabidopsis thaliana* are associated with changes in cytoplasmic pH at the tip

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ABSTRACT

Phosphorus availability regulates root hair elongation in *Arabidopsis thaliana* by controlling tip growth rate and growth duration, but little has been described on the effects of nitrate and ammonium. We hypothesized that changes in cytoplasmic pH at the tip caused by the elevation of phosphate, nitrate, or ammonium would be associated with changes in tip growth. The effects of phosphorus, ammonium, and nitrate availability on root hair pH and tip growth rate were investigated using quantitative ratio fluorescence microscopy and image analysis. Growing *Arabidopsis* root hairs were loaded with the pH sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF); neither root hair growth rate nor dye sensitivity to pH were disrupted by loading. Growing root hairs had a tip pH of 7.3. Tip pH was manipulated by the addition of fusicoccin, vanadate, propionic acid, acetic acid, or indole-1 acetic acid. Increasing or decreasing tip pH decreased elongation rate. Restoration of the tip pH by intracellular buffering systems either restored growth rate or stopped the decline in root hair growth rate. Treatment of root hairs with 1 mM nitrate triggered a simultaneous increase of tip pH and decrease in elongation rate; addition of 1 mM ammonium triggered a simultaneous decrease in pH and elongation rate. Root hairs grown in (?) or (?) had a similar tip pH, but treatment of low-phosphorus root hairs with 500 μ M resulted in an acidification of tip pH and a reduced elongation rate. We conclude that elongating root hairs maintain a homeostatic tip pH for maximum growth, and that high nitrate, ammonium, and phosphorus availability suppress root hair elongation by decreasing the cytoplasmic pH gradient.

INTRODUCTION

Phosphorus acquisition is an important concern for agriculture throughout the world, in diverse areas such as Africa (Krasilnikoff et al. 2003), India (Dwivedi et al., 2003), and Brazil (Lardy et al., 2002). Because of the importance of improved phosphorus acquisition efficiency, recent studies have sought to characterize important aspects of plant growth which can increase phosphorus uptake. Some of these morphological factors include adventitious rooting (Miller et al. 2003), aerenchyma (Fan et al. 2003), basal root angle (Bonser et al. 1996), and root length, root density, and root hair length (Krasilnikoff et al. 2003). Root hair length and density are both increased under phosphorus stress (Bates and Lynch, 1996; Ma et al. 2001a) Ma et al. (2001b) described a modeling approach to quantify the importance of root hair length and density in phosphorus uptake from a 'depletion zone' around the root cylinder, and longer root hairs are thought to give plants an advantage in phosphorus acquisition (Bates and Lynch, 2001).

In *Arabidopsis*, we have observed that over a physiologically valid range of external phosphorus concentrations, root hair length is regulated by phosphorus availability (Bates and Lynch, 1996). Low phosphorus availability increases final hair length by increasing both the rate and duration of hair elongation, which suggests that phosphorus stress accelerates metabolic activity in the elongating hair. Regulation of root hair elongation by phosphorus is a rapid, localized response, is specific for phosphorus, and may be mediated by auxin (Bates and Lynch, 1996). Auxin has many effects on root hairs. It has been shown to induce cortical microtubule randomization, which induces

root hair initiation when root hairs of *Lactuca sativa* are exposed to pH=4.0, and this is promoted by ethylene (Takahashi et al. 2003), and root hairs whose growth has been inhibited by hypapherine, which is produced by the ectomycorrhizal fungus *Pisolithus tinctorius*, are restored to normal elongation by the addition of IAA (Ditengou et al. 2000). While the effects of low phosphorus on root hair growth have been described, there exists at his time no explanation for the phenomenon.

Less emphasis on specific the specific effects of nitrate and ammonium on root hair growth. Foehse and Jungk (1983) reported that deficiency in phosphate and nitrate stimulated the growth of root hairs, and that this effect was not observed with deficiencies in potassium, ammonia, calcium, or magnesium. Ammonia has been shown to alkalinize the cytoplasm of root hairs in maize and rice; and this was attributed to the GS/GOGAT reaction (Kosegarten et al. 1997). The restoration of cytosolic pH was attributed to the intracellular pH stat proposed by Davies (1973, 1986). However, elongation rate was not measured in these experiments. Nitrate (McClure et al. 1990) and phosphate (reviewed in Smith et al. 2003) are cotransported with H⁺, so their uptake would affect cytoplasmic pH.

The mechanism of tip growth has been reviewed in Ridge (1996). Intracellular calcium gradients are essential for root hair tip growth (Wymer et al, 1997) and external calcium influx has been measured in growing *Arabidopsis* root hairs (Schiefelbein et al, 1992), but there is not a strict correlation between the intracellular calcium gradient and root hair growth (Halperin et al. 2003). An alternative candidate is pH in the cytoplasm. In *Sinapis alba* root hairs, regulation of intracellular pH by ion transport and proton pumping suggests that pH may also be an important mechanism in regulating tip growth

(Bertl and Felle, 1985; Felle, 1987; Felle, 1994). Membrane depolarization in root hairs induced by auxin treatment caused a decrease in both cytoplasmic pH and calcium (Tretyn et al, 1991). Further studies with *Sinapis alba* root hairs show that elevated cytoplasmic calcium at the tip is needed for normal tip growth. In the same study, tip pH values indicated a slight acidic pH gradient but these values were not significantly different than pH values in the base of the cell; however, the study showed that tight regulation of overall cytoplasmic pH is essential for cell growth (Herrmann and Felle, 1995). Acidification of the cell wall has been shown to be important for root hair initiation and cytoplasmic pH is temporarily elevated from 7.3-7.7, but growing root hairs maintained a pH of 7.3 (Bibikova et al. 1998). Sodium chloride stress also had no effect on cytoplasmic pH in root hairs (Halperin et al. 2003). These findings are consistent with an earlier report that a pH gradient is not required for root hair growth (Parton et al., 1997)

Homeostatic regulation of cytoplasmic pH in other plant systems suggests that pH is an important mediator in cell functions. *Acer pseudoplatanus* cells respond to artificial cytoplasmic acidification with a recovery to more normal pH values. Removal of the acid load led to an alkaline adjustment in cytoplasmic pH (Guern et al, 1986). In *Catharanthus roseus* cells, cotransport of inorganic phosphate and protons caused a cytoplasmic acidification that did not recover to control values until the phosphorus was exhausted from the medium (Sakano et al, 1992). In the forementioned studies on cytoplasmic pH regulation, induction or inhibition of proton pumps in both the plasmalemma and tonoplast has been the putative mechanism of pH regulation. In corn root hairs, a method to study vacuolar proton pumping with quantitative ratio

fluorescence has been developed (Brauer, 1995) and gibberellic acid caused a decrease in vacuolar pH in barley aleurone cells (Swanson and Jones, 1996).

The objectives of this research were to (1) measure cytoplasmic pH in growing *Arabidopsis* root hairs, (2) evaluate the relationship of tip pH with root hair elongation rate, and (3) test the hypothesis that ammonium, nitrate, and phosphorus availability affect root hair elongation in association with changes in cytoplasmic pH. We measured cytoplasmic pH using esterified BCECF (BCECF-AM) because it has been used in a wide range of studies, e.g. *Arabidopsis thaliana* root hairs (Halperin *et al.* 2003), *Catharanthus roseus* cells in cell suspension culture (Sakano *et al.*, 1992), maize root hairs (Brauer *et al.*, 1995) and barley aleurone protoplasts (Swanson and Jones, 1996).

RESULTS

BCECF Calibration and Measuring Cytoplasmic pH in Root Hairs

As shown in figure 1, the emission intensity of BCECF at 535nm increased with increasing solution alkalinity when excited with 505nm light. Conversely, BCECF emission intensity was independent of pH when excited at 439nm light. BCECF is an effective ratio probe for the measurement of cytoplasmic pH in plant cells because the emission intensity shift at the pH sensitive wavelength is most pronounced between pH 6 and pH 8 and the isobestic point is at a measurable intensity (Haugland, 1996). Figure 2 shows growing *Arabidopsis* root hairs loaded with 1 μ M BCECF-AM, the membrane-permeant acetoxymethyl form of the fluorochrome. Dividing the pixel values of the

505nm image by the 439nm image gives a ratio image that indicates true intracellular pH. Examination of the 439nm image, a rough indicator of dye loading, shows that BCECF fluorochrome is distributed throughout the entire root hair and is not restricted to either the cytoplasm, vacuole, or other sub-cellular compartment. Based on fluorescent signal intensity and the healthy growth rate of *Arabidopsis* root hairs, we found that the optimal loading concentration of BCECF-AM was between 1-2 μ M. Dye loading at a concentration of 5 μ M or greater provided a stronger signal but disrupted normal cytoplasmic streaming and stopped tip growth. Disruption of normal cell function at high loading concentrations coincided with sequestering of the dye into the vacuole. BCECF-AM concentrations lower than 1 μ M did not provide a strong enough signal to obtain an accurate pH measurement. Optimal dye loading is also a function of root hair age. We found that very young as well as terminally elongated root hairs load less (approximately 10% of maximum) than root hairs that were 50% to 75% of their terminal length.

Sensitivity of BCECF to pH was not affected by intracellular loading, as shown by comparison of *in situ* and *in vitro* calibrations in figure 3A. In figure 3B, ratio images of root hairs loaded with BCECF-AM and equilibrated internally with nigericin, sodium azide, and various external pH values show how emission intensities translate to colors and how those colors are ascribed to a continuous color scale. In this method, esterases in growing root hairs cleave BCECF-AM into fluorescent BCECF before nigericin is added; therefore, fluorescence is measured only from BCECF inside the root hair and not from uncleaved BCECF-AM in the external medium. The *in situ* calibration curve was used for all experimental pH measurements.

Variation of intracellular pH of growing root hairs with respect to morphology

Figure 2 and Figure 4A show results from typical dual excitation ratio experiments on growing *Arabidopsis* root hairs. Comparison of the ratio images with their corresponding light images (figure 2). Cytoplasmic distribution and the position of the nucleus are visible under conventional bright field and phase contrast microscopy. Figure 4A shows the pseudocolor ratio image of a typical growing root hair and figure 4B shows an intensity linescan of that image with pixel values converted to real pH values from the *in situ* standard curve (figure 3A). Growing root hairs have an cytoplasmic pH of 7.3 at the tip of the cell. While there appear to be variations in pH in the nucleus and the vacuole (corresponding to the red and blue areas in figure 1, respectively), these variations can also result from variation in dye accumulation since the dye can accumulate in different organelles. To confirm our measurements of cytoplasmic pH, BCECF-dextran (10,000 molecular weight) was loaded into growing root hairs by iontophoretic microinjection. The dextran-conjugated form of BCECF is too large to cross cellular membranes, therefore, the dye will be trapped into the cellular compartment that it is injected. Figure 5 shows the dual excitation ratio images of a growing root hair being microinjected with BCECF-dextran. Impalement at the junction of two root hair cells yielded the highest success rate for cytoplasmic loading and continued cell viability. A successful injection was defined as cells where fluorescence at the 439 excitation wavelength could be measured in the cytoplasm and not in the vacuole and where growth rate remained high after impalement with the micropipette. Data from cells where the dye loaded into the vacuole or where impalement disrupted tip growth were discarded. Results from our microinjection experiments confirm the cytoplasmic pH distribution

found with BCECF-AM. The values obtained for tip pH are consistent with those obtained from pressure injection of a dextran conjugate of BCECF (Halperin *et al.* 2003). Thus, at least with respect to cytoplasmic pH at the tip, potential problems from sequestration associated with iontophoretic injection (Knight *et al.*, 1994; Read *et al.* 1992) did not inhibit accuracy.

A neutral tip pH is essential for root hair growth

To study the importance of the observed cytoplasmic pH in root hair tip growth, we treated growing root hairs with compounds that have been shown to alter intracellular pH in other studies (Kurkdjain and Guern, 1989 for review). Figures 6A and 7 show representative ratio, tip pH, and growth rate results of a root hair naturally reaching its terminal length. As the root hair approaches a growth rate of zero, maximum tip pH decreases, and normal cytoplasmic distribution is replaced by rapidly streaming strands of cytoplasm characteristic of cytoplasmic strands surrounding the vacuole in the base of the cell. Weak membrane-permeant organic acids, such as propionic acid, can be used to decrease cytoplasmic pH (Felle *et al.*, 1992; Gibbon and Kropf, 1994). In figures 6B and 8, 5 μ M propionic acid (PA) caused an initial decrease in tip pH followed by a recovery, overshoot, and final decline in tip pH. The propionic acid treatment caused an initial decrease in root hair growth rate followed by an increase and terminal decline. The change in growth rate closely follows the change in tip pH with the exception that during the recovery phase, pH will overshoot to values more alkaline than the pre-treatment pH and growth rate never fully recovers to the pre-treatment growth rate. Acetic acid treatment caused an immediate decline in both pH and growth rate with no recovery (data

not shown). Fusicocin (FC) has been used in plants to stimulate the plasma membrane H^+ -ATPase and alkalinize the cytoplasm (Arif and Newman, 1993; Assmann and Schwartz, 1992; Bertl and Felle, 1985). Figures 6C and 9 show that $10\mu M$ FC caused an initial alkalization of tip pH followed by a drop to a neutral pH. Growth rate, on the other hand, responded to fusicocin with an initial decrease followed by a recovery phase and then a terminal drop. In this case, the transient recovery of growth rate takes place as the tip pH passes through pre-treatment pH values during the drop to a neutral pH. Treatment with $100\mu M$ vanadate, an inhibitor of proton pumping (Dunlop and Gardiner, 1993; McClure et al, 1990), caused both tip pH and root hair growth rate to decrease (Figure 10).

It has been suggested that auxin increases cell growth by stimulating the plasma membrane H^+ -ATPase (Tretyn et al, 1991). In previous studies, we found that $1nM$ IAA increased root hair length in *Arabidopsis* (Bates and Lynch, 1996). We tested this concentration of IAA on the pH gradient in root hairs. Figure 11 shows the pH and growth rate response in root hairs treated with $1nM$ IAA. IAA caused a slight initial increase in tip pH followed by an oscillating pattern. Growth rate also shows an oscillating pattern where the overall growth rate increases.

Nitrate (NO_3^-) and ammonium (NH_4^+) have also been used to manipulate cytoplasmic pH through biochemical utilization (McClure et al, 1990; Ullrich and Novacky, 1990). NO_3^- has been shown to cause an initial cytoplasmic alkalization whereas NH_4^+ causes initial cytoplasmic acidification (Raven, 1986). Figures 6D and 12 show that $8mM$ NO_3^- ($1mM$ greater than in culture media) caused cytoplasmic alkalization and a decrease in growth rate. Figures 6E and 13 show that $4mM$ NH_4^+

(1mM greater than in culture media) caused a similar but less pronounced pattern in tip pH and growth rate as propionic acid.

Cytoplasmic pH may mediate the response of root hair growth to phosphorus availability

Low phosphorus availability increases root hair length in *Arabidopsis* by increasing both growth rate and growth duration (Bates and Lynch, 1996). We found that the pH distribution in high-phosphorus and low-phosphorus-grown root hairs are similar; however, we tested the effect of externally applied phosphorus on cytoplasmic pH and root hair growth rate. Figures 6F and 14 show that 500 μ M phosphorus caused a decrease in both pH and growth rate. Recovery of pH coincides with a recovery of root hair growth rate. As with PA and NH_4^+ , an alkaline overshoot in pH leads to a terminal decline in growth rate.

Figure 15 shows the relationship of root hair tip pH and growth rate taken from all treatments. Root hairs have an optimum tip pH of approximately 7.3 for maximum growth. Deviation from the optimum pH, either naturally or by treatment effect, decreases tip growth rate.

DISCUSSION

Cytoplasmic pH in Growing Root Hairs

Our studies show that growing root hairs have a cytoplasmic pH that is more alkaline at the tip of the hair. Initial alteration of the tip pH intensity through biochemical or biophysical processes decreases root hair growth rate. Regulation of cytoplasmic pH in response to initial pH changes causes the growth rate to either stop declining or to begin rising again. In most treatments, a final decline in both growth rate and tip pH is measured. These results suggest that an established pH gradient is essential for root hair tip growth and that the cell attempts to regulate the cytoplasmic pH gradient after a perturbation in the system. The final decline phase of pH and growth rate represents the dissipation of the pH gradient and suggests that the cells have a limited capacity to buffer against perturbations in pH.

The plasma membrane H^+ -ATPase is stimulated by fusicocin and inhibited by vanadate. These treatments represent a biophysical alteration in cytoplasmic pH (the transport of protons across membranes). Although both treatments initially decrease the growth rate of the cell, fusicocin initially increases tip pH by increasing the extrusion of protons from the cell and vanadate decreases the pH gradient by inhibiting proton pumping. This suggests that tip pH homeostasis is maintained at least partially by proton pumping.

Like fusicocin, it has been suggested that IAA increases proton extrusion by stimulating the plasma membrane H^+ -ATPase. In our system, 1nM IAA initially increases tip pH which supports this theory. Contrary to the other treatments that led to a terminal decline in growth rate, IAA induced an oscillation in growth rate that progressively increased growth rate values. In *Zea mays* coleoptiles, 1 μ M IAA caused

oscillations in both intracellular calcium and pH (Felle, 1988). In a previous study (Bates and Lynch, 1996), we showed that 1nM IAA increased root hair length. Data from the present study suggest that the effect of IAA on tip pH may account for increased root hair length. The difference in effective IAA concentration on root hairs and coleoptile cells may represent the difference in tissue receptivity to exogenous auxin.

At a pH of 5.7, the pH of our growth media, propionic acid and acetic acid are protonated and are able to cross biological membranes into a cytoplasmic pH of approximately 7.2. By nature of their dissociation constants, these acids release their protons inside the cells and acidify the cytoplasm. In our system, propionic acid and acetic acid both decrease root hair tip pH and root hair growth rate. In the case of propionic acid, the root hair was able to restore tip pH and growth rate even while still in the presence of propionic acid. It appears that the mechanism for restoring pH, such as proton pumping or organic acid formation, is strong enough to alkalinize the cytoplasm. The final decrease in tip pH and growth rate in both propionic acid and acetic acid treatments represents a disintegration of tip pH suggesting that there is a limit to the cells' buffering capacity.

Nitrogen metabolism in plants contributes largely to the cation-anion relationship in plants. Since approximately 70% of the cations and anions taken up by the roots is either NH_4^+ or NO_3^- , an imbalance of these ions alters the cation-anion uptake ratio and consequently leads to changes in cytoplasmic pH that the plant must buffer against (Raven, 1986; van Beusichem et al, 1988). Assimilation of NH_4^+ in our root hairs caused an initial decrease in tip pH because of proton release and caused an initial decrease in root hair growth rate; these results contrast with those of Kosegarten et al. (1997). It

should be pointed out that in the latter study, maize root hairs and rice root hairs responded differently to NH_4^+ , suggesting that this may be a species-specific response. The recovery and final decline in pH and growth rate with NH_4^+ treatment is similar to the propionic acid treatment which may suggest similar control mechanisms. In contrast to NH_4^+ , NO_3^- assimilation leads to the production of OH^- in the cytoplasm (Raven, 1986). We observed that treatment of root hairs with NO_3^- caused an increase in tip pH and a decrease in growth rate. As tip pH is restored to normal values, growth rate begins to recover but then finally drops as tip pH dissipates.

Taken together, these treatments demonstrate root hair reaction to perturbations in cytoplasmic pH. Artificial treatments (PA, FC, Vanadate, Acetic acid) cause rapid and strong changes in pH and growth rate. As a result, these treatments may overstimulate the cellular pH buffering system, causing further fluctuations in pH from which the cell cannot recover. Treatments with naturally occurring agents (NO_3^- , NH_4^+ , IAA) cause more gradual changes in pH and growth rate and may place less demand on the intracellular buffering system. All treatments, with the exception of IAA, cause a terminal decline in growth rate. We interpret this as a saturation of cell homeostatic capacity.

The Cytoplasmic pH Gradient and Phosphorus Availability

Our previous studies showed that root hairs subject to low phosphorus availability grew longer and faster than root hairs subject to high phosphorus availability. Now that we have established that growing root hairs have a narrow range of tip pH associated with tip growth rate, we tested the effect of phosphorus availability on the pH gradient of

root hairs. Addition of phosphorus to low-phosphorus root hairs caused an initial decrease in cytoplasmic tip pH and tip growth rate. This supports the theory that phosphate anions co-transport into root cells with protons (Sakano et al, 1992). As with propionic acid and NH_4^+ , tip pH recovers and turns alkaline before the gradient dissipates. Similarly, growth rate levels off as tip pH recovers and then finally declines as the gradient dissipates. This shows that acidification of the cytoplasm by a variety of acid loads induces a buffering mechanism regardless of the type of acid load. Addition of phosphorus to high-phosphorus root hairs caused no change in tip pH or growth rate (data not shown). It has been suggested that phosphorus deficient media increase the catio-anion ratio which may decrease cytoplasmic pH (Schjorring, 1986). In the case of root hairs, this may decrease the steady-state cytoplasmic pH gradient. We found that untreated low-phosphorus root hairs had a slightly higher tip pH than high-phosphorus root hairs; however, this difference proved to be statistically insignificant. Therefore, it is possible that any growing root hair (high-phosphorus or low-phosphorus) maintains an optimum tip pH. High-phosphorus grown root hairs expend more energy working against the pressure of increased phosphate/proton symport to maintain homeostatic tip pH and membrane potential than low-phosphorus grown root hairs. As a result, there is less energy available in a high-phosphorus root hair for other cell growth functions and tip growth rate is declined. Future research in this area may yield much information on the relationship of root hair growth and phosphorus availability. Since nitrate deficiency also stimulates root hair growth (Foehse and Jungk, 1983), a similar mechanism may be functioning.

We conclude that growing *Arabidopsis* root hairs maintain a homeostatic tip pH that is measurable with quantitative ratio fluorescence imaging. Changing cytoplasmic pH through proton pumping, acid loading, ion transport, or nutrient assimilation, disrupts pH homeostasis and decreases tip growth rate (figure 15). Root hairs possess a mechanism that buffers against changes in pH and tries to maintain the pH gradient homeostasis. The inhibition of root hair growth in high-phosphorus grown root hairs may be mediated by the effect of phosphate/proton symport on cytoplasmic pH.

METHODS

Plant Material

Arabidopsis (RLD ecotype, Ohio State University Arabidopsis Biological Resource Center) plants were grown in medium consisting of half-strength nutrient salts (Johnson et al., 1957), myo-inositol (0.55mM), MES (2.5mM), sucrose (2% w/v), and Phytigel (Sigma, 0.2%) dissolved in Millipore-filtered water and adjusted to a pH of 5.7 with dilute potassium hydroxide. For media deficient in phosphorus, ammonium sulfate was substituted for ammonium phosphate. High-phosphorus and low-phosphorus media were autoclaved at 15 PSI for 15 min. at 121 °C and then 2 ml was pipetted onto No. 1, 24 x 60 mm cover slips (Corning Glass Works). Seeds were surface sterilized by washing successively in 70% ethanol for 30 seconds, 20% commercial bleach and Triton X-100 for five minutes, and then rinsed three times in sterile water. Seeds were sown on the solidified media, one seed per cover slip, placed in a sterile Petri dish, and sealed with parafilm. Seeds were incubated for 8 to 16 days at a 45° angle in a plant culture room

with constant light ($40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation) and temperature (25°C). By incubating plants at a 45° angle, root growth occurred toward the bottom surface of the gel, minimizing the working distance between the sample and microscope objective. Surrounding the root with gel provided a homogeneous environment for the growing root hair cells in terms of temperature, humidity, nutrient concentration, mechanical impedance, etc.

Loading of Root Hairs with BCECF-AM

A 1mM stock solution of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Molecular Probes, Eugene, OR) was dissolved in DMSO. Aliquots of the stock were diluted in Millipore water to a concentration of $1\mu\text{M}$. Sections of solidified media on the cover slips surrounding the root hair elongation zone were cut away to minimize gel volume; however, care was taken not to disturb the root hairs. BCECF-AM was applied directly to the top and cut surfaces of the gel as well as the coverslip. In growing root hairs, dye loading could be measured after 15 minutes and loading stabilization could be measured after 30 minutes.

Microinjection of Root Hairs with BCECF-dextran

Stock BCECF-dextran (10,000 MW, Molecular Probes) was dissolved in Millipore water to a concentration of $100\mu\text{M}$. Borosilicate glass tubes (1B150F-4, World Precision Instruments, New Haven, CT) were pulled to an injection diameter unresolvable under 600X magnification, approximately 2 microns (PC-84 pipette puller, Sutter Instruments, Novato, CA). BCECF-dextran was backloaded into the glass

micropipettes and iontophoretically microinjected into root hairs with a current between 5-10nA (Microiontophoresis Dual Current Generator 260, World Precision Instruments, Sarasota, Fl). Impairment and dye injection took place at the epidermal junction of two root hair cells and current was applied until fluorescence intensity was equivalent to 1 μ M BCECF by monitoring the pH independent wavelength.

Epifluorescence Microscopy and Image Acquisition

Loaded root hairs were viewed on a Nikon Diaphot inverted microscope (Garden City, NJ) with a long working distance dry Fluor X60 objective (Nikon, Japan, numerical aperture 0.70). Excitation wavelengths of 505 \pm 8-nm and 439 \pm 8-nm were isolated from a 75 watt xenon arc lamp by two scanning monochrometers (Photon Technology International, South Brunswick, NJ). Excitation and emission wavelengths were separated by a 515-nm dichroic mirror and BCECF fluorescence was isolated through a 535 \pm 25-nm bandpass filter (Nikon, Japan). Fluorescence emission intensity was increased at both excitation wavelengths by a Hamamatsu Photonics C2400 Intensifier (Japan, setting=9.0). Fluorescence and bright field images were collected with a Hamamatsu Photonics XC-77 charged-couple device (CCD) camera (Japan). UniBlitz excitation shutters and CCD camera were controlled by Metamorph Software (Universal Imaging Corporation, West Chester, PA).

Ratio Image Analysis

Metamorph software was used for ratio analysis of raw data image pairs. Background fluorescence at both excitation wavelengths was omitted prior to image

acquisition by adjusting the intensifier to a setting of 9.0 and the CCD camera to a setting of 100 white level and 50 black level. There was no detectable autofluorescence at these intensifier and camera settings. 535-nm emission images were collected by successively illuminating loaded root hairs with 505-nm excitation light and 439-nm excitation light. Pixel values of the 505-nm images were divided by the pixel values of the 439-nm images to give ratio images. Ratio values were then converted to pH values with the *in situ* calibration curve (described below).

In vitro and in situ Standard Curves

For the *in situ* ratio pH calibration, root hairs were loaded with BCECF-AM (as previously described). After dye loading stabilization, internal and external pH was equilibrated by incubating root hairs with sodium azide (0.05%), nigericin (100 μ M), and media strongly buffered at pH values from 5.5 to 8.5. After ten minutes of equilibration, ratio images were taken (as previously described) and a calibration curve was generated from an average of five root hairs at each pH level. For an *in vitro* pH calibration, BCECF-salt was dissolved in pH buffered solution (final concentration = 1 μ m) and emission intensities were measured with a cuvette-based photodetector system (Photon Technology International). Ratio values were computed directly from emission intensity values.

Treatment Reagents

Propionic acid, acetic acid, potassium phosphate (dibasic-phosphorus source), potassium nitrate (NO₃⁻ source), calcium nitrate (NO₃⁻ source), and ammonium sulfate

(NH₄⁺ source) were obtained from Fisher. Fusiccocin, IAA, and sodium vanadate were obtained from Sigma. All reagents were brought to their treatment concentrations by dilution in deionized water.

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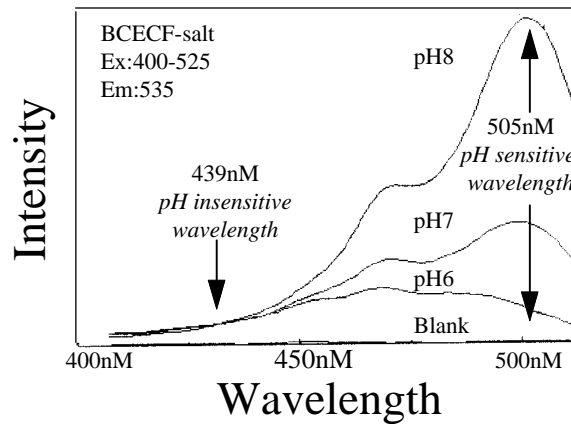


Figure 1. Emission fluorescence response of BCECF to pH.

Emission intensity of BCECF-salt at 535nm when illuminated with light from 400-520nm. When excited at 505nm, the emission intensity is responsive to pH. Emission intensity is independent of pH when excited at 439nm. Arrows indicate excitation wavelengths used for experiments.

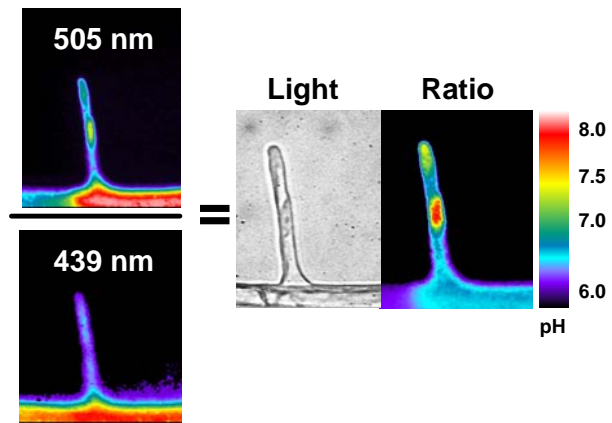


Figure 2. BCECF Loading and Ratio Image Acquisition in an *Arabidopsis* Root Hair.

Root hairs were loaded with 1 μ M BCECF-AM and images were collected by ratio fluorescence microscopy. Dividing the pixel intensities of the 505-nm image by the 439-nm image gives a ratio image pseudocolor encoded for pH.

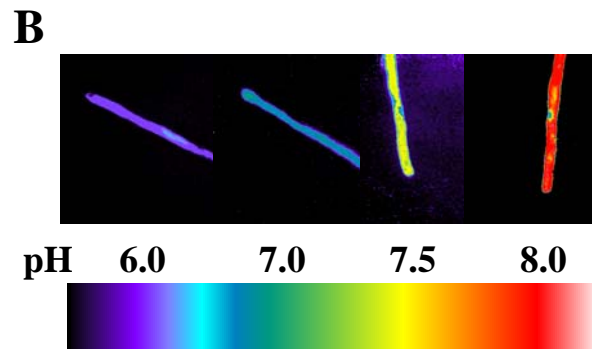
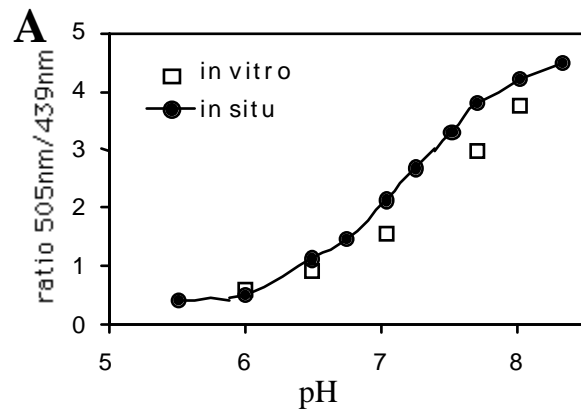


Figure 3. *In situ* and *in vitro* calibration curves of BCECF.

(A) *In situ* and *in vitro* response of BCECF fluorescence to pH .

(B) *In situ* ratio images of root hairs equilibrated with various external pH values. After loading growing root hairs with BCECF-AM, the roots were incubated with nigericin, sodium azide, and growth media buffered at a desired pH. Similarity in the *in situ* and *in vitro* data shows that the response of BCECF to pH is unaffected in root hairs.

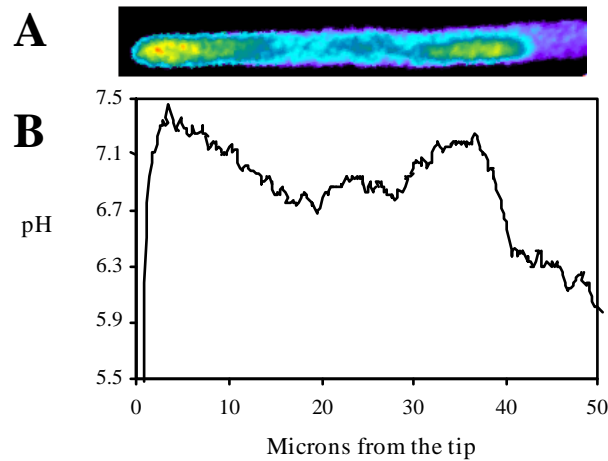


Figure 4. Example of Linescan Measurement of pH in a Growing Root Hair

(A) Ratio fluorescence image of growing *Arabidopsis* root hair loaded with BCECF.

(B) Fluorescence intensity linescan of the same root hair with intensity values converted to pH values by the calibration curve.

After loading with BCECF-AM, the 505nm/439nm images were collected and the ratio image calculated. Each point on the linescan is an average pH across the width of the root hair at that point. Both images show the tip pH of 7.3. Because of dye mobility through intracellular membranes, pH values subapical to the tip are considered approximate.

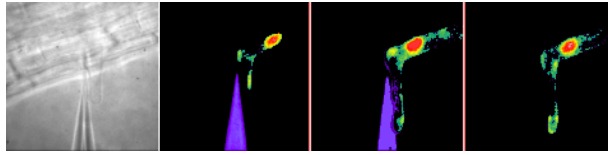


Figure 5. Microinjection of BCECF-dextran.

Root hairs were microinjected with $1\mu\text{M}$ (final concentration) BCECF-dextran (10,000 MW). The light image shows the root hair at the time of impalement. Ratio images show tip pH during and after dye loading.

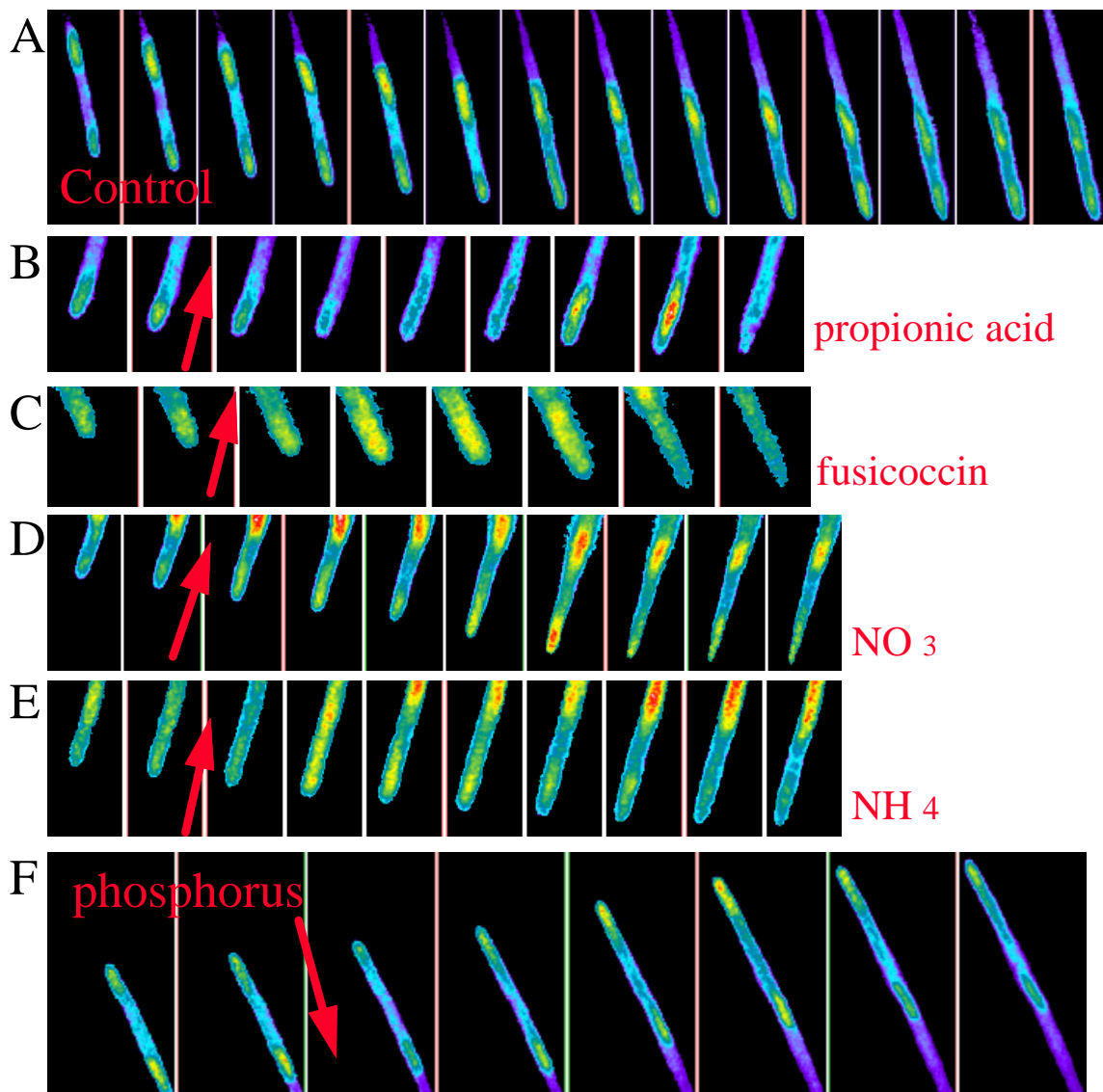


Figure 6. Manipulation of the Tip pH and Root Hair Growth Rate

(A) Naturally terminating root hair.

(B) Root hair treated with 5 μ M propionic acid

(C) Root hair treated with 10 μ M fusicoccin

(D) Root hair treated with 1 mM additional nitrate

(E) Root hair treated with 1 mM additional ammonium

(F) Root hair treated with 500 μ M phosphorus

All root hairs were loaded with BCECF-AM and ratio images (505nm/439nm) were acquired at five minute intervals. Arrows indicate the time the treatment was added to the growth media. Deviation from homeostatic pH values led to decreased elongation rate.

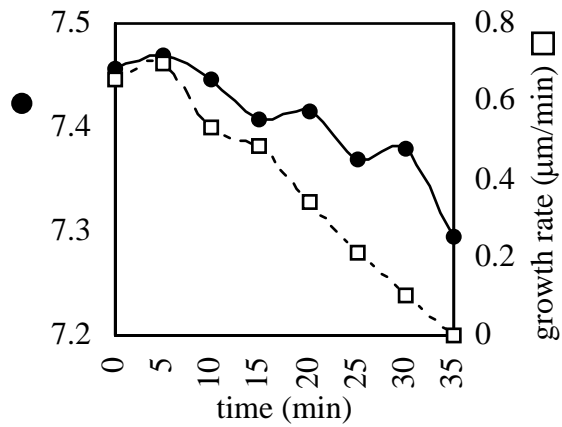


Figure 7. Tip pH and Growth Rate of Naturally Terminating Root Hair.

Images of BCECF-AM loaded root hairs were acquired and analyzed every five minutes. Natural termination of tip growth as seen by a steady decrease in growth rate coincides with a decrease in maximum tip pH (representative data, n=10)

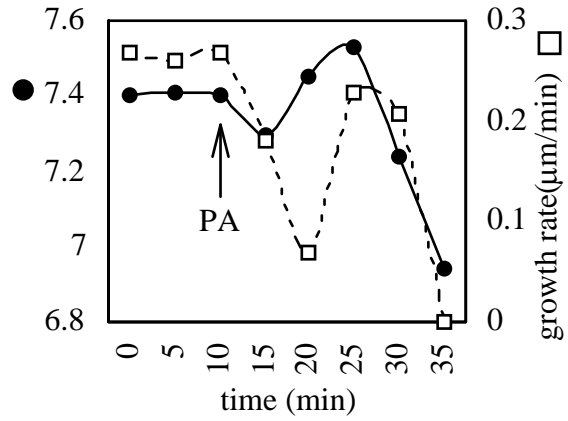


Figure 8. Effect of Propionic Acid on Root Hair Tip pH and Growth Rate.

5 μ M propionic acid (PA) was added to the growth media of growing root hairs. The vertical line represents the time at which propionic acid was added (representative data, n=10).

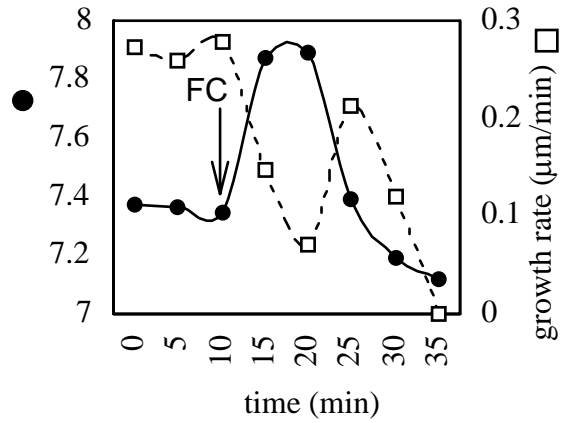


Figure 9. Effect of Fusicoccin on Root Hair Tip pH and Growth Rate.

10μM fusicoccin (fc) was added to the growth media of growing root hairs. The vertical line represents the time at which fusicoccin was added (representative data, n=10).

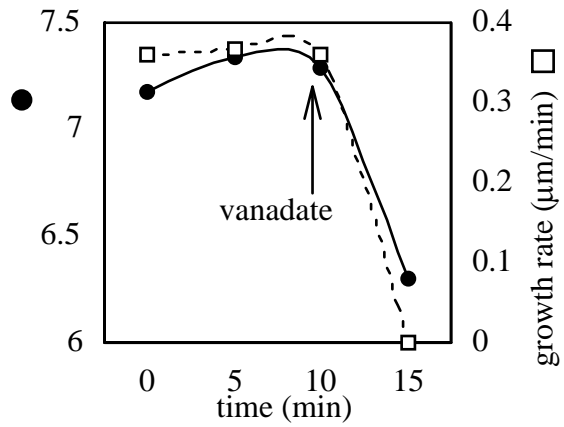


Figure 10. Effect of Vanadate on Root Hair Tip pH and Growth Rate.

100µM vanadate was added to the growth media of growing root hairs. The vertical line represents the time at which vanadate was added (representative data, n=5).

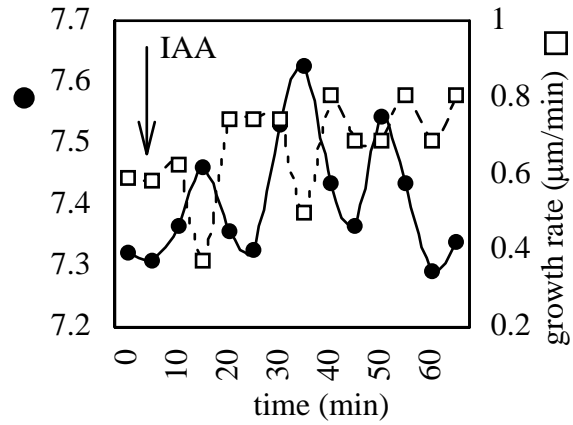


Figure 11. Effect of Indole-3 Acetic Acid on Root Hair Tip pH and Growth Rate.

1nM Indole-3 Acetic Acid (IAA) was added to the growth media of growing root hairs. The vertical line represents the time at which IAA was added (representative data, n=5).

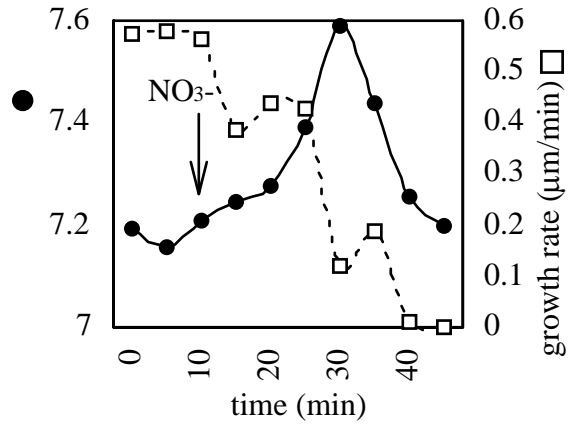


Figure 12. Effect of Nitrate on Root Hair Tip pH and Growth Rate.

1mM additional nitrate (KNO_3) was added to the growth media of growing root hairs. The vertical line represents the time at which nitrate was added (representative data, $n=10$).

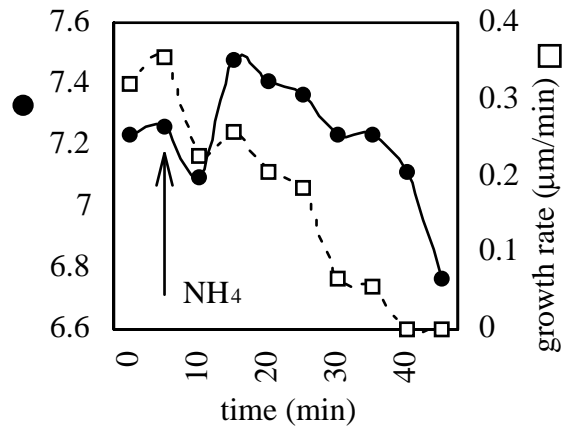


Figure 13. Effect of Ammonium on Root Hair Tip pH and Growth Rate.

1mM additional ammonium ((NH₄)₂SO₄) was added to the growth media of growing root hairs. The vertical line represents the time at which ammonium was added (representative data, n=10).

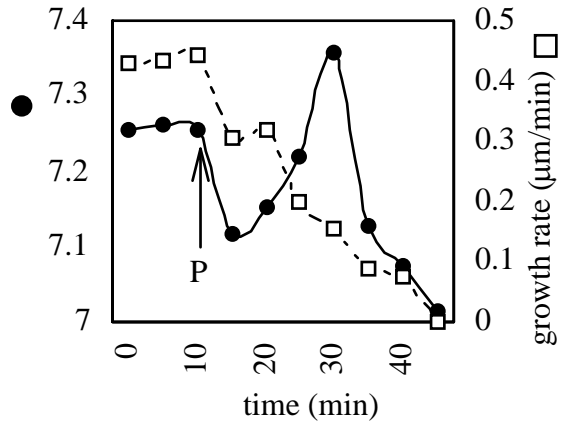


Figure 14. Effect of Phosphorus on Root Hair Tip pH and Growth Rate.

500 μ M additional phosphorus (KH_2PO_4 , dibasic) was added to the growth media of growing root hairs. The vertical line represents the time at which phosphorus was added (representative data, n=20).

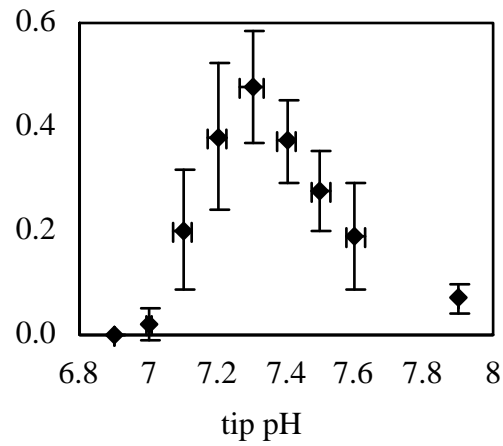


Figure 15. Cytoplasmic Tip pH and Growth Rate of Root Hairs

pH and growth rate data was combined from all root hair treatments. From pH 7.1 to 7.6, each point is the mean of 25. From pH 6.9, 7.0, and 7.9, each point is the mean of 5 (bars = \pm standard deviation).

Table I. pH Characteristics of Growing *Arabidopsis* Root Hairs

Measurement	Average \pm St.Dev.
Maximum pH of gradient	7.31 \pm 0.20
Minimum pH of gradient	6.72 \pm 0.35
Length of gradient (μm)	13.1 \pm 5
Nuclear pH	7.56 \pm 0.20
Distance of nucleus from tip (μm)	29.0 \pm 5
Vacuolar pH	5.73 \pm 0.25
Growth rate ($\mu\text{m}/\text{min}$)	0.40 \pm 0.22

Analysis of linescans from a population of growing root hairs on different plants gives representative root hair pH distribution. All root hairs were between 50%-75% of their terminal length making their growth rate approximately 50% of maximum (n=70).