Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in Arabidopsis thaliana

Tatiana N. Bibikova1, Tobias Jacob1,2, Ingo Dahse2 and Simon Gilroy1,*

1Department of Biology, The Pennsylvania State University, 208 Mueller Lab, University Park, PA 16802, USA
2Friedrich Schiller University, Institute of Biochemistry and Biophysics, Department of Biophysics, Philosophenweg 12, 07743 Jena, Germany
*Author for correspondence (e-mail: sxg12@psu.edu)

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SUMMARY

Morphogenesis in plants is characterized by highly regulated cell enlargement. However, the mechanisms controlling and localizing regions of growth remain essentially unknown. Root hair formation involves the induction of a localized cell expansion in the lateral wall of a root epidermal cell. This expanded region then enters a second phase of localized growth called tip growth. Root hair formation therefore provides a model in which to study the cellular events involved in regulating localized growth in plants. Confocal ratio imaging of the pH of the cell wall revealed an acidification at the root hair initiation site. This acidification was present from the first morphological indications of localized growth, but not before, and was maintained to the point where the process of root hair initiation ceased and tip growth began. Preventing the wall acidification with pH buffers arrested the initiation process but growth resumed when the wall was returned to an acidic pH. Cytoplasmic pH was found to be elevated from approximately 7.3 to 7.7 at the initiation site, and this elevation coincided with the acidification of the wall. Preventing the localized increase in cytoplasmic pH with 10 mM butyrate however did not inhibit either the wall acidification or the initiation process. In contrast, there was no detectable gradient in pH associated with the apex of tip growing root hairs, but both elevated apoplastic pH and butyrate treatment irreversibly inhibited the tip growth process. Thus the processes of tip growth and initiation of root hairs show differences in their pH requirements. These results highlight the role of localized control of apoplastic pH in the control of cell architecture and morphogenesis in plants.

Key words: Arabidopsis thaliana, Confocal ratio imaging, Cell wall pH, Cytosolic pH, Root hair, Trichoblast

INTRODUCTION

Root hairs are projections from epidermal cells of the root that have been proposed to play critical roles in water and nutrient uptake, and in anchoring the plant in the soil (reviewed by Ridge, 1995). Only certain cells (‘trichoblasts’; Leavitt, 1904) within the root epidermis are capable of undergoing differentiation into root hairs. The patterning of such differentiation is highly regulated. Thus the trichoblasts of Arabidopsis are limited to a series of 8 cell files precisely located at the intersection of two cortical cells (e.g. Dolan et al., 1993).

To produce a root hair, a trichoblast undergoes 2 genetically distinct processes: root hair initiation and tip growth of the initiated hair (Ridge, 1995). During initiation, a highly localized bulge is produced in the outer surface of the trichoblast (Leavitt, 1904). The production of this bulge represents a transition from diffuse longitudinal growth of the epidermal cell to highly localized and tightly regulated growth from the side of the cell. Once initiated the root hair commences tip growth where deposition of new cell wall material is confined to the expanding tip of the developing hair, leading to the elongated hair-like morphology (Schnepf, 1986).

Research on the tip growth of pollen tubes, algal rhizoids, fungal hyphae and root hairs suggests that a localized, highly focused gradient of cytoplasmic Ca2+ is a strong candidate for the spatial information determining the direction of growth (e.g. Bibikova et al., 1997; Felle and Hepler, 1997; Holdaway-Clarke et al., 1997; Malho and Trewavas, 1996; Wymer et al., 1997). However, no localized increase in cytoplasmic [Ca2+] could be observed to precede or predict the site of root hair initiation. Similarly, although the Ca2+-channel blocker verapamil leads to inhibition of tip growth, root hair initiation was unaffected (Wymer et al., 1997). Thus localized changes in cytoplasmic [Ca2+] seem intimately involved in tip growth, but are less convincing as central determinants of the initiation process.

Changes in both cytoplasmic and apoplastic pH are alternative candidates for determinants of localized growth. Acidification of the cytoplasm has been associated with the localized growth of Pelvetia rhizoids (Kropf and Gibbon, 1994) and Funaria protonemata (Demkiv et al., 1994), although in

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fungi, pollen tubes (Fricke et al., 1997; Parton et al., 1997) and tip growing root hairs (Herrmann and Felle, 1995), no correlation between apical growth and pH gradients could be found. Wall acidification also occurs in cells showing diffuse growth (Mulkey and Evans, 1981), where the low pH may activate proteins involved in wall relaxation (McQueen-Mason et al., 1992). Although such ‘acid-growth’ does not explain all aspects of the sustained elongation of plant cells, the limited wall extension associated with the initiation process of root hairs may be consistent with some degree of acid-based wall relaxation. Additional evidence for a role of apoplastic pH in plant morphogenesis arises from the report that local application of the expansin proteins thought to mediate acid-based wall relaxation causes bulging of the leaf primordia in tomato (Flemming et al., 1997). This localized control of tissue expansion may have been sufficient to trigger the developmental program of the primordia and induce leaf formation.

We therefore tested whether changes in cytoplasmic or wall pH were associated with the localized growth events associated with the initiation process of root hairs of Arabidopsis thaliana. We report that cytoplasmic pH increases, and wall pH decreases at the root hair initiation site. Prevening the change in apoplastic, but not cytoplasmic pHe, reversibly arrests the process of root hair initiation. Although disruption of cytoplasmic and apoplastic pH inhibited tip growth, no distinct gradient in cytoplasmic pH could be detected associated with the growing tip of these cells.

MATERIALS AND METHODS

Plant material and measurement of growth kinetics

Seedlings of Arabidopsis thaliana (Columbia ecotype) were grown as previously described (Wymer et al., 1997). Plants were used 4-7 days after planting, at which time the roots were 1-3 cm long. Growth kinetics were monitored on a Nikon Diaphot 300 microscope (Nikon inc., Melville, NY, USA) using a 40× dry, Nikon, 0.7 numerical aperture objective and differential interference optics as described previously (Wymer et al., 1997). Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fluorescent dyes were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Ratiometric measurement of cell wall pH

Root hair wall pH was monitored by pseudo-ratiometric confocal imaging of the fluorescence from the pH-sensitive dye NERF-Cl, conjugated to a 10 kDa dextran, and the pH insensitive dye Texas Red conjugated to a 10 kDa dextran (Taylor et al., 1996). Both dextran-conjugated dyes were infiltrated into the epidermal cell walls of the root by incubating the root in 100 μM of the dextran dyes for 30 minutes. Wall pH was then imaged on the stage of a Zeiss axiovert microscope attached to a LSM410 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA), using a 40× 0.75 numerical aperture, dry objective. Pseudo-ratio images were collected using excitation from the 488 and 543 nm lasers of the confocal microscope and emission collected at 515-540 nm (NERF) and >590 nm (Texas Red). Each frame represents a single 8 second scan of the laser. Pseudo-ratio images were then constructed by dividing the pH-dependent fluorescence from the NERF with the pH-independent fluorescence from Texas Red. Thus the ratio images of tip growing cells also reflected cytoplasmic pH. Wall pH was then imaged on the stage of a Zeiss axiovert microscope and imaged as described above for NERF/Texas Red using excitation from the 488 and 543 nm lasers and the emission collected at 515-540 nm (BCECF) and >590 nm (rhodamine). Each frame represents a single 8 second scan of the laser. Pseudo-ratio images were then constructed by dividing the pH-dependent fluorescence from the BCECF with the pH-independent fluorescence from the rhodamine, as described previously for calcium green/rhodamine imaging (Bibikova et al., 1997). This pseudo-ratiometric approach was found to provide a reliable measure of the cytoplasmic pH. Results from pseudo-ratiometric analysis of BCECF dextran/Texas Red dextran gave identical results to pseudo-ratiometric analysis with BCECF dextran/rhodamine dextran; therefore only the BCECF/rhodamine data is presented. In vitro calibration of the BCECF/rhodamine was performed using 10 μM BCECF dextran/rhodamine dextran, 100 mM KCl, 1 mM MgCl2, 1 mM ATP, 10 mM HEPES buffered to pH 6-8. In situ calibration was performed using 10 μg/ml nigericin and 100 mM extracellular KCl (Fricke et al., 1997). However, in 80% of cells tested, the in situ calibration failed to elicit a reliable, sustained change in cytoplasmic pH. The reason for this variability is unknown. Therefore due to the reproducibility of the in vitro calibration and the low numbers of microinjected cells available for in situ calibration, the values for cytoplasmic pH presented were calibrated according to the in vitro calibration. When successful, the in situ calibration agreed with the in vitro calibration by >90%. Despite such caveats about the calibration procedure it is generally thought that the fluorescent pH-sensitive dyes are accurately reflecting changes in pH (Fricke et al., 1997). Tip growing root hairs proved more difficult to microinject than initiating root hairs and due to the larger volume of their cytoplasm required more dye to be injected to lead to a reliable signal for ratio analysis. The maximal signal attainable from dextran-conjugated microinjected dyes in the tip growing root hairs was 50% of that from the initiating root hair. Injecting more dye led to a failure to maintain tip growth. Thus the ratio images of tip growing cells also appear more ‘noisy’ than those of initiating cells, but still accurately reflect cytoplasmic pH.

Bright-field images were also taken at each experimental time point using the transmission detector of the confocal microscope and the 633 nm He/Ne laser attenuated to 10% with neutral density filters.
RESULTS

Imaging of wall pH in Arabidopsis roots

To test the role of cell wall pH in the root hair initiation process, we imaged the fluorescence of the dextran-conjugated dyes, NERF and Texas Red infiltrated into the trichoblast wall. We then determined wall pH by pseudo-ratio imaging, monitoring the pH-sensitive fluorescence of NERF and comparing this to the pH-insensitive fluorescence of Texas Red (Taylor et al., 1996). As these dextran-conjugated dyes leaked from the cell wall within 10-20 minutes when the roots were washed with the dye-free medium, experiments were conducted with the indicator dyes still present in the medium around the root. We therefore used the optical sectioning ability of the confocal microscope to distinguish medium fluorescence from that of indicator in the epidermal cell wall. Unfortunately the need for continued dye presence in the medium precluded imaging pH in the exposed walls of the tip growing root hair. Therefore analysis of wall pH was limited to the initiation process.

Fig. 1 shows that taking an optical section approximately 2 µm below the surface of the epidermis resolved fluorescence from dye in the wall from the background fluorescence of the medium. Texas Red dextran (Fig. 1B) and NERF dextran (Fig. 1C) were similarly loaded into the apoplasmic space of trichoblasts in the root hair zone of the root and were excluded from the cytoplasm for as long as we have observed (12 hours). For comparison, Fig. 1E shows an equivalent region of a root stained with fluorescein diacetate (FDA), a dye that accumulates in the cytoplasm (Huang et al., 1986). The cell walls in this picture appear as dark regions that fail to accumulate the dye. Exclusion of the Texas Red and NERF dextran conjugates from the cytoplasm, and maintained localization to the wall, was observed in cells from the end of the elongation zone through to the mature region of the root (Fig. 1 and data not shown). This localization of indicator allowed us to monitor wall pH from just before root hair initiation (elongation zone cells), to past the end of the initiation process (mature region of the root).

We next ensured that infiltration of dextran-conjugated dye and subsequent fluorescence imaging did not affect root growth or root responses. The growth rate of roots loaded with the dextran-conjugated dyes and subjected to confocal imaging (201±15 µm/hour, n=7) was similar to unloaded controls (218±18 µm/hour, n=10). In addition, loaded roots showed identical morphology and kinetics of the gravitropic response (Blancaflor et al., 1998) when compared with unloaded controls (data not shown). The kinetics of root hair initiation and growth, and trichoblast and root hair morphology were also unaffected (see below). We take these as indications that the loading of the cell walls with dextran-conjugated dyes and subsequent ratio imaging, was neither perturbing root growth nor altering the process of root hair initiation.

Calibration of the NERF/Texas Red ratio to wall pH

We then determined that the wall located dyes were still pH responsive. The NERF/Texas Red signal was calibrated in situ and in vitro. Fig. 2A shows an in vitro calibration performed using 10 µM NERF/Texas Red dextran, 100 mM KCl, 1 mM MgCl₂, 10 mM of appropriate pH buffer (pH 4-5, dimethylglutarate (DMGA) or citrate; pH 5-6.5 MES; pH >6.5 HEPES) buffered to a range of pHs from 3 to 6. In situ calibration was made using dyes infiltrated into cell walls of roots that had been killed by freeze thawing and the metabolic activity in the cytoplasm inhibited by 1 hour incubation in ice cold ethanol. The measurements from these dead roots were made to assess the responsiveness of the dyes in the wall environment isolated from potential effects of the living root cell protoplast. For in vivo calibration, roots were loaded with the dextran-conjugated dyes and extracellular pH buffered using 50 mM of the appropriate pH buffer (Fig. 2B). Below 50 mM MES, HEPES, citrate or DMGA buffer concentration, the pseudo-ratio imaging indicated no reproducible change in cell wall pH (data not shown). However, Fig. 2B shows that at 50 mM pH buffer, wall pH changed in parallel to the pH set by the extracellular buffer system in the range 4.5 to 6.0. We attribute this requirement for high buffer levels to the strong natural pH buffering in the cell wall. Thus the NERF/Texas Red ratio was found to be linearly dependent on pH in the range 3 to 6 in the in situ calibration in walls of dead and living roots and in the in vitro calibrations (Fig. 2A). These three methods of calibration all showed an identical change in ratio of 0.27-0.29/pH unit over the linear pH 3 to 6 range. The ratio values were insensitive to Ca²⁺ (0-50 mM), K⁺ (0-200 mM) and Cl⁻ (0-200 mM) in vitro (data not shown).

When the root was incubated in pH buffer, all regions of

![Fig. 1. Cell wall localization of dextran-conjugated NERF and Texas Red.](image-url) Roots were perfused with NERF dextran and Texas Red dextran. (A) A bright-field image of cells shown in B and C. Fluorescence from Texas Red dextran (B) and NERF dextran (C) in the cell walls of the root hair initiation zone was imaged using confocal microscopy. The confocal optical section shown is approximately 2 µm from the root surface. (D) A bright-field image of cells of the root hair initiation zone stained with fluorescein diacetate (FDA) (E). Note the cell wall staining in B and C and cytoplasmic staining and exclusion from the cell walls in E. The bottom right panel shows a magnified view of the regions indicated in B-E. i, initiation site; m, fluorescence from medium; tr, trichoblast; v, vacuole. Representative views of more than 50 roots. Scale bar, 20 µm.
the wall showed an similar ratio value at the same buffered pH, including the walls at the initiation site (Fig. 2B). This uniform in vivo calibration was observed in the walls of trichoblasts throughout the initiation process, suggesting that different regions of the wall were not modifying the responsiveness of the NERF/Texas Red indicator system to pH. Although we have attempted to calibrate the responsiveness of the dyes in their intact wall setting, the absolute pH values quoted should be viewed as approximations of wall pH.

As expected, treatment with 100 nM of the H\textsuperscript+\textquotesingle-ATPase activator fusococcin led to a reduction in wall pH of between 0.5 and 1 pH unit (data not shown), consistent with the wall localized NERF/Texas Red responding to physiologically relevant changes in wall pH. In addition, when pH buffer was applied to the root, a change in wall pH was detectable (Fig. 2C), indicating that the ratio imaging approach was capable of detecting changes in wall pH. Thus the NERF/Texas Red ratio imaging approach appeared valid for monitoring changes in pH along the length of the cell as root hair initiation proceeded.

**Local wall acidification is associated with root hair initiation**

Figs 2D and 3 show that as trichoblasts enter the zone of root hair initiation, the pH of the trichoblast cell wall away from the initiation site (middle in Fig. 3A,B) increases to approximately pH 6 over 40-60 minutes. However, a region of ≤pH 5 is maintained at the site of root hair initiation. The pH of this initiation region drops to 4.5 over the initial 30 minutes of root hair initiation, which corresponds to the period of bulging of the wall that characterizes the initiation phase of root hair formation. When this initiation process was stopping and the root hair was switching from initiation to tip growth (approximately 40-60 minutes after the wall bulging had commenced (Fig. 3C), the locally reduced pH at the initiation site increased to the pH 6 found in the rest of the wall (Figs 2D, 3B).

Close inspection of the ratio images of wall pH in Fig. 2D shows some heterogeneity in wall pH of approximately ±0.2 pH units around the mean pH value for any region of the wall. The source of this variability is unknown. It does not arise from noise due to a low signal strength from the pH sensing dyes and is also not evident in the images of the strongly pH buffered walls in Fig. 2B. This variability may reflect natural microdomains of altered apoplastic pH due to the complex structure of the wall.

**Manipulating apoplastic pH alters root hair initiation and tip growth**

To determine whether the lowered wall pH maintained at the root hair initiation site was involved in the initiation events or merely coincided with them, we attempted to inhibit acidification of this wall region by buffering the apoplastic pH. We therefore applied the 50 mM HEPES, MES and DMGA buffers found to effectively alter wall pH in the in situ calibration shown in Fig. 2B. The growth rate shown in Fig. 4A was calculated 30 minutes after initiation had commenced but is representative of the effect of altering external pH at all times during the initiation process. When apoplastic pH was buffered between

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**Fig. 2.** Calibration of the NERF dextran/Texas Red dextran wall pH measurements. (A) The pH dependency of the NERF/Texas Red ratio was calibrated against pH in vitro using 1 μg/ml of each dextran, 100 mM KCl, 1 mM MgCl\textsubscript{2}, and 10 mM of appropriate pH buffer (pH 4-5: citrate or DMGA; pH >5 MES). The in situ calibration was obtained by infiltrating the dyes into cell walls of roots killed by freeze thawing and incubation in ice cold ethanol. The roots were then incubated in 50 mM of pH buffer for the in vitro calibration and mean ratio values calculated from regions of the wall encompassing >1000 pixels using IPLabs image analysis software. Mean ± s.e.m., n>12. Linear regression was calculated using Microsoft Excel and least squares fitting, R\textsuperscript{2} in situ, 0.93; in vitro, 0.97. (B) Ratio images of the in vivo calibration of the NERF/Texas Red ratio. The dyes were infiltrated into cell walls of living roots which were then incubated in 50 mM of the appropriate pH buffer for 15 minutes. Note the homogeneous change in wall pH throughout the wall, including the initiation site (i). (C) The cell walls of a trichoblast were loaded with NERF and Texas Red dextrans and pH determined by confocal pseudo-ratio imaging. Wall pH was then altered by applying 50 mM MES, pH 6.0 and imaged after 5 minutes. Note the rapid alkalinization of the cell wall induced by this change in apoplastic pH treatment. (D) Shows ratio images of cell walls at the indicated time after root hair initiation. Note the decrease in pH in the cell wall at the initiation site (i) and increase in wall pH by 55 minutes after initiation has commenced. Wall pH has been pseudo-color coded according to the inset scale. The dark region to the side of the initiation site in the 55 minute image is due to interference with the wall pH imaging by the long root hair that has formed and grown out over this region of the cell wall. The images in D are from roots grown in standard growth medium (1/2 strength Epstein’s medium, 2.3 mM MES pH 5.7) without the supplemental pH buffers used in the calibrations shown in A-C. Representative of more than 10 roots. Scale bar, 10 μm.
4.5 and 5.5 the average rate of the wall bulging, indicative of the initiation process, increased as the pH was lowered until at pH 4.0 growth was inhibited (Fig. 4A). This was probably due to a cytotoxic action of prolonged exposure to pH 4.0.

When the same buffer systems were used to alter wall pH to ≥5.5, root hair initiation was inhibited. This inhibition occurred irrespective of the stage of initiation previously reached by the root hair (Fig. 4A and data not shown). Thus, for example, a trichoblast where the lateral cell wall had not yet begun to bulge, but where the root hair would be predicted to start to emerge in the next few minutes, showed no bulging at an external buffered pH of 7.0. Initiating root hairs were arrested at the stage of initiation they had reached when the wall pH was elevated (Figs 4A, 5). Upon returning the extracellular pH from 7.0 to pH 5.0 (still in the MES/HEPES...
buffer system) initiation growth recovered and resumed normal root hair formation with no detectable lag (Fig. 5B,C). This indicates that the inhibition of initiation was not due to the buffer used, but most likely to the pH set by that buffer. The resumption of growth of initiating root hairs was observed when the exposure to high extracellular pH was less than 30-45 minutes (Fig. 5C). Longer periods of high pH treatment led to a failure of all initiating root hairs to resume growth. In contrast tip growth was inhibited when extracellular pH was increased to >7.0 and irreversibly arrested at pH ≥7.5 irrespective of the time of treatment at elevated pH (Fig. 4B and data not shown). At pH <4.5 tip growth was inhibited and 25% (n=54) of growing root hairs burst. No root hairs that had ceased to elongate before the pH 4.0 treatment were observed to burst (n=10 separate roots).

Altering wall pH with 50 mM MES/HEPES at pH 7.5 led to no detectable change in cytoplasmic pH monitored by BCECF dextran/rhodamine dextran ratio analysis (n=5, see below).

**Measuring cytoplasmic pH in Arabidopsis root hairs**

We next sought to determine if the local changes in wall pH in trichoblasts initiating a root hair were mirrored by changes in cytoplasmic pH. Developing trichoblasts were therefore microinjected with the pH sensing fluorescent dye BCECF dextran and the pH insensitive dye rhodamine dextran, and pH monitored throughout the initiation and tip growth process. As found previously, microinjected, dextran-conjugated dyes were excluded from the vacuole and remained in the cytoplasm for as long as we have observed (>8 hours) and did not alter the process of root hair formation and growth (Bibikova et al., 1997; Wymer et al., 1997). Fig. 6A shows an in vitro and in vivo calibration of the BCECF/rhodamine ratio versus pH and indicates that this ratiometric analysis should accurately resolve pH in the expected cytoplasmic range (pH 7.0-8.0). Fig. 6C,D shows that in atrichoblasts or trichoblasts prior to root hair emergence, cytoplasmic pH was homogeneous at approximately 7.2. However, a localized increase in pH to 7.7±0.2 (n=11) was seen to be associated with the site of initiation in trichoblasts. This alkalinization formed concurrently with the first morphological indications of bulging in the trichoblast cell wall (Figs 6C, 7). The alkaline pH was maintained throughout the bulging process. Cytoplasmic pH in atrichoblasts did not detectably change during the time when initiation was occurring in adjacent trichoblasts (Figs 6D, 7C). Using this confocal ratiometric approach to monitor cytoplasmic pH, tip growing root hairs showed no detectable, marked pH gradient (>0.1 pH unit) either acidic or alkaline, associated with the growing root hair apex (Fig. 6G). Root hairs that had stopped growing showed a similar, homogeneous cytoplasmic pH of 7.1-7.2 (Fig. 6I).

**Altering cytoplasmic pH inhibits tip growth but not root hair initiation**

We next asked if the alkalinization in cytoplasmic pH was involved in the initiation process or simply coincident with it. We applied the weak acids propionic acid and butyric acid to alter cytoplasmic pH and determined the effect on initiation kinetics. Butyric and propionic acid gave identical results, therefore only the data from butyric acid experiments is presented. Disruption of cytoplasmic pH may also be accomplished using weak bases such as NH4Cl (Kossegarten et al., 1997). However, this was found to be inapplicable in

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Fig. 5. Reversibility of the inhibition of root hair initiation by elevated extracellular pH. Root hair growth was monitored as the extracellular pH was changed from pH 5.0 to 7.5 and then returned to 5.0 using 50 mM MES/HEPES buffer. (A) Images of a time course of initiation in root hairs maintained at pH 5.0, and (B) shows the effect of an excursion to pH 7.5 over the indicated times. Note the inhibition of initiation at pH 7.5 and recovery on return to pH 5.0. (C) The kinetics of the resumption of growth of a root hair as pH was changed from 5.0 to 7.5 and back to 5.0. Note the failure to resume growth when the pH 7.5 treatment is longer than 30 minutes. Results are representative of >25 root hairs on 8 separate roots per treatment. Bar, largest s.e.m.
these root hair experiments as the weak bases require elevated external pH, e.g. pH 7.0, for action. This elevated external pH could alter initiation and tip growth kinetics due to effects on apoplastic pH. In addition weak bases have been shown to alter vacuolar pH in root hairs (Brauer et al., 1997).

Butyrate at 10-15 mM inhibited tip growth but failed to prevent root hair initiation (Fig. 8). Growth of the main root axis was also unaltered by up to 10 mM butyrate treatment (control 218±18 μm/hour; 10 mM butyrate for 2 hours, 201±11 μm/hour, n=10). Thus at 10 mM butyrate, the root continued to elongate and a series of initiated root hairs accumulated along the root but these failed to start tip growth (Fig. 8A-C). The effects of 10 mM butyrate were reversible for the initiation process but irreversible in tip growing root hairs. Thus initiated root hairs recommenced growth after perfusion of the root with butyrate free medium for >6 hours (data not shown). Ten mM butyrate dissipated the local elevated cytoplasmic pH at the initiation site (Fig. 8D-F) but did not affect the locally acidic wall at the initiation site (Fig. 8G). Ten mM butyrate caused no statistically significant change in the cytoplasmic pH of tip growing root hairs (apical 5 μm of control: 7.3±0.13; butyrate: 7.17±0.2 pH units, P>0.05 t-test).

At higher concentrations of butyrate (e.g. 20 mM) root hair elongation was inhibited, and elongation growth of the main root axis was slowed (51±30 μm/hour, n=10). However, even under these conditions, the process of root hair initiation proceeded with no detectable alteration in kinetics or morphology of the initiating hair (data not shown).

DISCUSSION

Root hair initiation involves an epidermal cell changing its growth habit from a diffuse longitudinal growth in the elongation zone of the root to highly localized lateral growth that will form the tip growing root hair. Initiation of the root hair and its subsequent tip growth are two independent phases of root hair formation that exhibit differences in the genes required and in their cell biological controls (e.g. Ridge, 1995; Wymer et al., 1997).

Although the bulging associated with root hair initiation is preceded by a nuclear migration (Meeks, 1985; Sato et al., 1995) and the cytoskeleton has been shown to become reoriented after the initiation process (Emons and Derksen, 1986), the molecular mechanisms underlying the reorientation of growth are unknown. We have found that the site of initiation is associated with an alkalinization of the cytoplasm and a local wall acidification. Preventing the local acidification of the cell wall by raising the extracellular pH with buffers stopped the initiation process. Importantly, this inhibition was reversible, suggesting the effect was not due to a cytotoxic action of the high external pH. The rapid reversibility of the inhibition of initiation elicited by high external pH also suggests that the pH responsive elements of the wall are stable. Wall polymer structure (Carpita and Gibeaut, 1993), enzyme activities (Fry et al., 1992; Nishitani et al., 1992),
effects on the levels of other ions e.g. Ca\(^{2+}\) (Arif and Newman, 1993) and channel activities in the plasma membrane (Hedrich and Dietrich, 1996) are all potential sites of action of altered apoplastic pH. Additionally, acid-induced growth has been linked to wall extensibility in roots (e.g. Pritchard, 1994), and proteins with acidic pH optima (expansins; McQueen-Mason et al., 1992) that can mediate wall relaxation of root cell walls.

**Fig. 7.** Changes in cytoplasmic pH in a trichoblast and atrichoblast during root hair initiation and tip growth. Trichoblasts and atrichoblasts were microinjected with dextran-conjugated BCECF and rhodamine, and cytoplasmic pH determined by confocal pseudo-ratio imaging. In an initiating root hair cytoplasmic pH measurements were taken at the initiation site or away from the initiation site in the middle of the cell. For tip growing root hairs, the pH measurements were made from the flank or tip, as indicated in (A). Average cytoplasmic pH in trichoblasts (B) and atrichoblasts (C) was calculated from the ratio images using IPLabs image analysis software using a 5-10 μm region of the cytoplasm as indicated in the insets in A. For the atrichoblast, pH at the initiation site represents the pH in the cytoplasm at the point where a root hair would be expected to emerge if the cell were a trichoblast. For tip growth, the measured region at the flank of the root hair was representative of the pH throughout the cytoplasm to the apical 10 μm region covered by the measurement of tip pH. tr, trichoblast. Results are mean ± s.e.m. (n=7, atrichoblasts; n=11, trichoblasts).

**Fig. 8.** The effect of 10 mM butyrate on root hair initiation and tip growth. (A) The root hair zone of a control root and (B) one treated with 10 mM butyric acid for 4 hours. Note the profusion of elongated (tip growing) root hairs of the control (□) and the accumulation of initiated root hairs that have failed to start tip growth (arrows) in the butyrate treated root. The elongated root hair in the butyrate treated root (❖) was already tip growing when the butyric acid was applied. Scale bar represents 50 μm. (C) The effect of butyrate on the kinetics of root hair initiation and tip growth. Roots were treated with (❖) or without (□) 10 mM butyric acid and the growth of the initiation point measured. Results are representative of more than 8 root hairs. Bar, largest s.e.m. (D) Bright-field image of E and F. (E,F) Ratio imaging of cytoplasmic pH in a trichoblast before (E) and after (F) treatment with 10 mM butyrate for 10 minutes. Note dissipation of elevated pH at the initiation site (i) in the butyrate treated root. Representative of 8 separate root hairs. (G) Wall pH monitored by NERF/Texas Red ratio imaging in a trichoblast treated with 10 mM butyrate before (E) and after (F) treatment with 10 mM butyrate for 10 minutes and then imaged at -5 and 20 minutes after the initiation process had commenced. The continued acidification of the wall at the initiation site (i) despite butyrate treatment. Cytoplasmic and wall pH have been color coded according to the inset scales. Representative of more than 8 individual roots.
have been isolated (Wu et al., 1996). Expansins have been cloned from Arabidopsis (Scherban et al., 1995) and would be predicted to show minimal wall relaxing activities at pH ≥7.0 (McQueen-Mason et al., 1992).

Localized cellular growth phenomena in Fucus and Pelvetia zygoites (Kropf and Quatrano, 1987, Kropf et al., 1995), Micrasterias (Holzinger et al., 1995) and Funaria (Demkiv et al., 1994) have also been linked to alterations in cytoplasmic pH. Interestingly, rhizobial nod factors alter root hair growth habit and have been shown to elicit an elevation in cytoplasmic pH in alfalfa root hairs (Felle et al., 1996). This elevation is coincident with the inhibition of apical growth and the induction of root hair deformation and curving that occurs during nodulation. These observations suggest that the increased cytoplasmic pH may play a role in these alterations of root hair growth form. In contrast, we have found that the cytoplasmic alkalinization at the root hair initiation site is not an absolute requirement for the initiation process to proceed. Thus blocking the increase in cytoplasmic pH with butyrate did not prevent initiation. Similarly, raising wall pH blocked initiation without affecting the locally elevated cytoplasmic pH at the initiation site.

The role of cytoplasmic pH in tip growth is also unclear. The pH-sensitive vibrating microelectrode has detected both influx (Jones et al., 1995) and efflux (Hermann and Felle, 1995) of protons at the growing tips of root hairs. In root hairs of Sinapis Alba impaled with pH selective microelectrodes no pH gradients were detected associated with tip growth (Hermann and Felle, 1995). We were also unable to detect a pH gradient associated with the growing tip of root hairs of Arabidopsis. Although we cannot exclude the possibility of changes below the spatial (≤1 μm) or temporal (≤1 second) resolution of our imaging equipment were not recorded. Assuming the lack of a pH gradient accurately reflects pH dynamics in the growing hair, the inhibition of tip growth caused by alteration of apoplastic pH or treatment with butyrate may well reflect disruption of the pH homeostasis required for the tip growth machinery to operate, rather than effects on a steady state pH gradient at the tip. A similar conclusion was drawn by Hermann and Felle (1995) for the effects of manipulating cytoplasmic pH on tip growth in root hairs of Sinapis Alba. In addition, we noted that at pH <4.5, growing root hairs often burst at the tip whereas root hairs that had previously stopped growing were unaffected. Lowering the extracellular pH to below 4.5 may weaken the pH dependent events regulating structure of the wall at the tip of the hair to the point where turgor causes bursting. The wall polymers at the tip of the non growing root hairs might then be modified to be insensitive to this pH change as a normal part of the mechanism whereby growth is arrested.

Whether the localized pH changes at the initiation site reflect localized changes in wall polymer structure and ion exchange capacities (Carpita and Gibeaut, 1997) or localized activation of the H+-ATPase or other H+ transporting activity is unknown. We have found agents that should alter H+-ATPase activity, such as vanadate and cyanide do inhibit the initiation process, but also rapidly (<30 minutes) inhibit root growth (data not shown). Such manipulations may be altering root hair initiation by disrupting other root processes rather than a direct action on the H+-ATPase at the initiation site. Therefore they do not provide reliable evidence for a role of the H+-ATPase in this process. However, a plasma membrane H+-ATPase has been immunolocalized in barley root hairs (Samuels et al., 1992) and root hairs show a detectable H+ current at their apex (Weisenseel et al., 1979; Jones et al., 1995). In addition, a H+-ATPase is localized to the apical plasma membrane of another tip growing system, pollen tubes (Obermeyer et al., 1992). Putative mechanisms for a localized activation of the H+-ATPase at the initiation site include reversible inhibition by submicromolar Ca2+ (Kinoshita et al., 1995) or Ca2+-dependent phosphorylation (Schaller and Sussman, 1988). Thus although our data suggest the potential activity of the H+-ATPase in the root hair initiation process, further studies are needed to precisely define its role.

Our results indicate apoplastic pH is intimately linked with localized growth of the initiating root hair. However, the root hair initiation process is a complex reorientation of growth that is likely to involve many interacting regulatory systems and localized wall modifications. Thus we found that simply reducing wall pH with a pH buffer did not trigger or accelerate the initiation process, nor did it lead to inappropriate bulging of the trichoblast wall. The nuclear migration that precedes initiation (Meeks, 1985; Sato et al., 1995) suggests the events determining the initiation site are laid down long before the first morphological indications of wall bulging. Consistent with this idea, we have been unable to detect a distinct pH change in either the wall or cytoplasm predicting the initiation site. However, although localized pH changes in the wall and cytoplasm do not appear to determine the initiation site, our results strongly suggest the pH changes in the wall are intimately involved in the machinery that translates the signals specifying the initiation site into the process of root hair emergence. In contrast, although we could not detect localized pH gradients associated with tip growth in root hairs, treatments that should disrupt apoplastic and cytoplasmic pH dynamics both inhibit the tip growth process. These results further support the genetic data (Ridge, 1995) that tip growth and root hair initiation are very different processes and suggest that control of both cell wall and cytoplasmic pH dynamics is essential in maintaining tip growth.

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