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RESEARCH PAPER

Sodium chloride reduces growth and cytosolic calcium, but does not affect cytosolic pH, in root hairs of *Arabidopsis thaliana* L.

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Abstract

The effects of salinity (NaCl) stress on growth, cytosolic Ca^{2+} gradients and cytosolic pH homeostasis of root hairs of *Arabidopsis thaliana* are assessed here. Neither cytosolic Ca^{2+} nor pH at the hair apex were significantly affected by 20 min exposure of up to 90 mM NaCl or of up to 5 mM extracellular Ca^{2+}. Exposure to increasing NaCl concentrations, up to 90 mM, for 2 d or 6 d reduced hair extension, and this inhibition was relieved by supplemental extracellular Ca^{2+}. Such extended salinity stress reduced the magnitude of the Ca^{2+} gradient in the apical 12 μm of hairs at all NaCl concentrations tested (up to 90 mM), including NaCl concentrations that did not reduce hair extension. The magnitude of the tip-focused gradient was also reduced in root hairs of plants grown with low (0.5 mM) extracellular Ca^{2+} when compared to those in 5 mM extracellular Ca^{2+}, regardless of the presence of NaCl. Up to 90 mM NaCl did not affect cytosolic pH of root hairs in any of the treatments. It is concluded that NaCl inhibition of root hair extension in the long term may operate via alterations in the tip-focused Ca^{2+} gradient that regulates root hair growth. However, NaCl-induced alterations in this gradient do not always lead to detectably altered growth kinetics. Short-term signalling events in response to NaCl may operate by a means other than altering Ca^{2+} at the root hair apex. Salinity stress in root hairs does not appear to be mediated by effects on cytosolic pH.

Key words: BCECF, cytosolic calcium, cytosolic pH, Indo-1, root hair, salinity, sodium chloride.

Introduction

Salinity stress is an important constraint to world agriculture, affecting 7% of the land surface of the earth and over 50% of irrigated land (Flowers *et al*., 1997). Salinity stress elicits complex effects on plant metabolism resulting from ion toxicity, water deficit, and nutrient imbalances (Pasternak, 1987; Werner and Finkelstein, 1995). It is likely that the phytotoxic action of NaCl is, in part, elicited by impairment of nutrient acquisition by roots. Although physiological responses of plants to salinity stress have been exhaustively researched, the effects of the various components of salinity stress on root hairs remain essentially unknown. For example, water stress has been shown to inhibit root hair growth, and to cause tip bulging in root hairs grown under aseptic conditions in Murashige–Skoog medium (Schnall and Quatrano, 1992). However, a study conducted in soil indicated that water stress actually enhanced root hair growth (Mackay and Barber, 1987).

Regulators of root hair growth range from genetic determinants (Foreman and Dolan, 2001) to phosphorus availability in the growth medium (Bates and Lynch, 1996; Ma *et al*., 2001), but several studies have also shown that an intracellular calcium gradient, focused on the root hair apex, is essential for root hair extension (Wymer *et al*., 1997; Bibikova *et al*., 1997; Felle and Hepler, 1997; Miller *et al*., 1997; de Ruijter *et al*., 1998). For example, the direction of root hair growth has been attributed to endogenous polarity and the direction of the Ca^{2+} gradient (Bibikova *et al*., 1997), and treatment of plants with verapamil, a putative Ca^{2+} channel blocker, also stopped root hair growth (Wymer *et al*., 1997). Similarly, root hair growth can be enhanced by increasing exogenous Ca^{2+},

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which increases the influx of Ca^{2+} at the tip and so presumably reinforces the growth-related Ca^{2+}-gradient (Herrmann and Felle, 1995).

Cytosolic pH has also been implicated in maintaining root hair growth (Herrmann and Felle, 1995; Bibikova *et al*., 1998; Bates, 1998). For example, changes in extracellular pH between 5 and 9 interrupted root hair growth, and agents that alter cytosolic pH (such as acetic acid and ammonium chloride) also inhibit root hair growth (Bibikova *et al*., 1998; Bates, 1998). It appears that tight control of cytosolic pH is important to maintain tip growth and, although an alkaline band has been reported to correlate with apical growth in pollen tubes (Feijo *et al*., 1999), there is no clear evidence for an internal pH gradient required for tip growth in root hairs (Herrmann and Felle, 1995; Parton *et al*., 1997; Bibikova *et al*., 1998). It is also likely that cytosolic Ca^{2+} and pH homeostasis are closely linked (Bush, 1993). Thus, disruption of growth via alterations in pH could be mediated through action on the Ca^{2+} gradient rather than a growth-related pH gradient.

The observations outlined above suggest that NaCl might alter root hair function through changing Ca^{2+} and/or pH dynamics in the growing root hair apex. Indeed, NaCl treatment has been noted to disturb Ca^{2+} nutrition of roots (Lynch and Läuchli, 1985), to cause a reduction in intracellular Ca^{2+} in root cells (Lynch *et al*., 1988; Cramer and Jones, 1996) and Ca^{2+} was lost from corn root protoplasts within minutes of application of 100 mM NaCl, most likely via NaCl effects on Ca^{2+} release from intracellular stores (Lynch *et al*., 1988). Lynch *et al*. (1989) also reported that NaCl treatments greater than 100 mM stimulated an elevation of Ca^{2+} in the cytosol, measured with the fluorescent Ca^{2+}-sensitive dye Indo-1. Taken together, these results suggested that NaCl stimulated a release of Ca^{2+} from organelles, which was then lost from the cell. Sodium chloride and water stress have also both been shown to reduce cytosolic Ca^{2+} in the apical cells of the primary root of *A. thaliana*, also within minutes (Cramer and Jones, 1996). However, in this study, a concurrent elevation of cytosolic Ca^{2+} was not observed, even though the amounts of NaCl used were comparable to the study by Lynch *et al*. (1989).

It is not clear if the alterations of cytosolic Ca^{2+} in response to NaCl action outlined above represent elements of a stress signalling system or an effect of toxicity. However, rapid elevations in Ca^{2+}, considered to be signalling events, have been observed in the roots of *Arabidopsis* plants which were exposed to NaCl (Kiegle *et al*., 2000). The Ca^{2+} spikes occurred within the first minute of application of stress and lasted for about 10–15 s. Interestingly, the signals are different from those obtained from other stresses, such as cold. The salt-related Ca^{2+} spikes are of short duration (s), and result in a large increase of Ca^{2+} in the cytoplasm. These measurements were made using plants expressing the transgenic Ca^{2+} reporter aequorin. Due to the inherently low aequorin signal from single cells (Knight *et al*., 1993), it was not possible directly to determine whether the root hairs contributed significantly to the spikes monitored by Kiegle *et al*. (2000). Thus, it is not yet known whether there is a rapid Ca^{2+} spike involved in stress signalling in root hairs exposed to NaCl.

The evidence for NaCl effects on cytoplasmic pH is conflicting. Katsuhara *et al*. (1989) reported that 100 mM NaCl caused an acidification of cytosolic pH of 0.10 units in the freshwater alga *Nitellopsis obtusa*, whereas Katsuhara *et al*. (1997) reported that over 200 mM NaCl had no effect on cytoplasmic pH in *Hordeum vulgare* L. root cells. A possible explanation of this difference is that the habitats of these species have different salinities and so the organisms may show markedly different salt tolerances. Sodium concentrations in rivers and lakes are typically below 20–30 mM (Horn and Goldman, 1994), but are greater than 40 mM in saline soils and at the surface of the root can be much greater (Marschner, 1986). There is clearly a need carefully to correlate the effects of NaCl on pH with detectable effects on growth in order to link cytosolic pH with potential phytoxic effects of the NaCl treatment.

It is hypothesized that the addition of external NaCl to root hairs will inhibit their extension rate, and will also lower cytosolic Ca^{2+} at the tip. Because a cytosolic Ca^{2+} gradient is so important for root hair growth, a correlation is also expected between the peak of the cytosolic Ca^{2+} gradient and extension rate. It is also hypothesized that additional external Ca^{2+} will minimize the effects of NaCl. The effects of NaCl on pH are not clearly predictable; NaCl may or may not alter cytosolic pH. There is a known Na/H antiporter in the plasma membrane of *A. thaliana* (Qiu *et al*., 2002), thus pH may decrease when root hairs are exposed to NaCl.

In this study, the effects of salt stress on root hairs were examined with respect to extension rate, intracellular pH, and intracellular Ca^{2+}. *Arabidopsis* plants were grown in 0.5 mM, 2.0 mM, or 5.0 mM Ca^{2+} and were treated with 0, 30, 60 or 90 mM NaCl for 2 or 6 d to examine long-term effects of salt stress on root hair growth. After 2 or 6 d, cytosolic pH and Ca^{2+} were measured with the use of fluorescent dyes, and hair extension rates were measured. To verify that NaCl did not affect measurements from the dyes by causing them to partition, the most extreme treatment (0.5 mM Ca and 90 mM NaCl, after 6 d) was microinjected with the dextran-conjugated forms of BCECF or Indo-1. The data were collected, and correlated. In a short-term experiment, plants were grown in medium containing 0.5, 2.0 or 5.0 mM Ca without NaCl, and the root hairs were viewed through a microscope. Liquid medium containing 0, 30, 60 or 90 mM NaCl was applied to the medium directly above the root hair being viewed. After 20 min, pH was measured via the fluorescent dye

BCECF. In order to view possible Ca^{2+} spikes, the root hair was acid-loaded with the fluorescent Ca^{2+} indicator Indo-1, and the region just below the apex was continuously monitored for 2 min by a photometer. To verify the results, root hairs were microinjected with the dextran-conjugated form of Indo-1. Because elevating external Ca^{2+} promotes root hair growth, it was reasoned that root hairs grown in elevated Ca^{2+} might grow even under the most saline conditions. It is reported that NaCl treatment does inhibit root hair growth and this inhibition is reduced by elevating extracellular Ca^{2+}. Sodium chloride treatments that alter root hair growth also reduce the cytoplasmic Ca^{2+} gradient at the root hair tip, but do not affect cytoplasmic pH. Unexpectedly, it was also noted that NaCl concentrations that have no detectable effect on hair extension also alter cytoplasmic Ca^{2+} levels, suggesting that altered growth and magnitude of the tip-focused Ca^{2+} gradient are not obligately linked.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* L. (ecotype Columbia) were sterilized with 95% (v/v) ethanol for 5 min, followed by 10% (v/v) NaOCl for 5 min, followed by rinsing in sterilized water. They were planted onto a sterile (autoclaved) solid medium consisting of 2% (w/v) sucrose, 0.25% (w/v) Phytagel (Sigma, St Louis, MO), 0.55 mM myo-inositol, 2.5 μM MES buffer (pH 5.5), 6 mM KNO_3, 1 mM $MgNO_3$, 0.4 mM K_2HPO_4, 2 μM KCl, 0.3 μM H_3BO_3, 0.1 μM $MnSO_4$, 0.1 μM $ZnSO_4$, 0.03 μM $CuSO_4$, 0.05 μM $NH_4H_2MoO_4$, and 3 μM FeEDTA. Calcium was added as $CaSO_4$ and its final concentration was adjusted according to the experimental treatment.

Two ml of the solid medium was spread onto a 24×60 mm coverslip (two coverslips per 15×100 mm round Petri dish: Fisher Scientific, Pittsburgh, PA), and allowed to harden in a sterile transfer hood for 1 h. One seed was placed on each coverslip. The seeds were germinated in the dark for 1 d, and then were exposed to constant light (40 μmol photons m^{-2} s^{-1} of photosynthetically active radiation provided by fluorescent lights) for the remainder of the experiment. After 3 d, the roots were 1 cm long, which was considered to be the standard length for the start of all experiments. During the entire period of growth, the Petri dishes were angled at 30° to encourage root growth towards the bottom of the gel.

Dye loading

Seedlings were acid loaded with Indo-1 (Teflabs Inc., Austin, TX) as described previously (Wymer *et al*., 1997). A 1 mM stock solution of 2′-7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxy-methylester (Teflabs Inc., Austin TX) in dimethylsulphoxide (DMSO) was diluted to a final concentration of 2.5 μM in growth medium and was added to the roots and allowed to load for at least 15 min. Alternatively, 10 000 kDa dextran conjugates of BCECF or Indo-1 (Molecular Probes Inc, Eugene, OR) were microinjected into root hairs as described previously (Bibikova *et al*., 1997).

Microscope and imaging equipment

To determine root hair growth rates, roots were observed with a Nikon SMZ-U stereomicroscope, and the images were captured with a Pulnix (Sunnyvale, CA) charged-couple device (CCD) camera and imported into Metamorph ver. 3.0 imaging software (Universal Imaging Company, West Chester, PA).

Fluorescence imaging of dye-loaded root hairs was carried out with a Nikon Diaphot inverted microscope (Garden City, NJ) with a 40× oil immersion, 1.3 numerical aperture, phase/fluor objective. For Indo-1 measurements, excitation of 355±5 nm was isolated from a 75 W xenon arc lamp by a scanning monochromator (Deltascan, Photon Technology International, South Brunswick, NJ). Excitation and emission wavelengths were separated by a 380 nm dichroic mirror and Indo-1 fluorescence was monitored through either a 405 nm or 485 nm (±20 nm) filter mounted in a filter wheel (Sutter Instrument Company, Novato CA). Images were collected using a Hamamatsu Photonics XC-77 CCD camera equipped with a Photonics C2400 Intensifier. Uniblitz excitation shutters and the CCD camera were controlled by Metamorph Software (Universal Imaging Corporation, West Chester, PA). Alternatively, Indo-1 was ratio imaged using a Zeiss LSM 410 confocal microscope as previously described (Wymer *et al*., 1997).

BCECF measurements using the Diaphot microscope system were taken under identical conditions to Indo-1 except that two excitation monochromators were used to alternately to deliver 439±5 nm and 505±20 3 nm light to a 515 nm dichroic mirror and emission was monitored using a 535±25 nm filter. Confocal ratio imaging of BCECF-dextran was performed as described previously (Fasano *et al*., 2001).

Ratio image analysis

Metamorph software was used for ratio analysis of raw data image pairs. Setting the intensifier to 7.0, and the analogue contrast to white level=51 and black level=27 eliminated any detectable autofluorescence signal from the root or root hairs.

To obtain measurements of the Ca^{2+} or pH distribution along the length of the apical 12 μm of the hairs, a transect was taken (5 pixels wide) along the centre of the root hair from the tip backwards. The gray scale intensity on this transect was measured at points corresponding to 0, 2, 4, 6, 8, 10, and 12 μm from the tip on each image of the ratio pair, and these values were used to calculate the ratio needed for Ca^{2+} or pH quantification using the standard curves described below.

Photometer measurements

For the rapid time-course measurements to monitor for Ca^{2+} spikes and transients, a PTI D104 microphotometer unit equipped with two 01610 detectors was attached to the side of the Diaphot microscope in place of the camera. The unit utilized a 455 nm dichroic beam-splitting mirror and a 405±20 nm and 485±20 nm emission filter. The opening of the iris was set to measure from a 5×5 μm square centred on the tip of the root hair such that the cytoplasm completely filled this opening without monitoring the edge of the hair. Measurements were taken for 130 s with 0.5 s sample rate.

Standard curves

In vitro and *in vivo* calibration of Indo-1 was performed as previously described (Wymer *et al*., 1997; Bibikova *et al*., 1998). The *in vitro* curve was used for converting ratio measurements into Ca^{2+} concentration because it lacked the additional variation resulting from the difficulties of accurately buffering Ca^{2+} inside of a plant cell (as also described in Wymer *et al*., 1997).

For BCECF calibration, root hairs were loaded with BCECF-AM and after 1 h the intracellular and extracellular pH was equilibrated by incubating the root hairs with sodium azide (0.05%, w/v), nigericin (20 μM), and either 20 mM 4-morpholinoethanesulphonic acid (MES) or 20 mM *tris*[hydroxymethylaminomethane] (Trizma base), pH values from 6.0 to 8.0. After 15 min of equilibration, ratio images were taken and measured as described above.

Short-term intracellular ion concentration experiments

This experiment utilized 3–4-d-old plants which had been grown in medium containing 0.5, 2.0 or 5.0 mM $CaSO_4$. Fifty seconds after measurement had begun, a 5 µl aliquot of medium supplemented with 0, 30, 60 or 90 mM Na^+ was added to the region that contained the hair being monitored. This volume of aliquot was determined to be the maximum that could be added without causing artefacts resulting from a potential change in focus as it was found that the growth gel moved upon the addition of larger volumes of liquid.

Long-term intracellular ion concentration and growth experiments

This experiment utilized 3–4-d-old plants grown in medium containing 0.5, 2.0 or 5.0 mM $CaSO_4$. Medium containing 0–90 mM NaCl was added and root hairs from the primary root were analysed for root hair extension, intracellular pH or the apical Ca^{2+} gradient 2 d (i.e. 5–6-d-old plants) or 6 d (i.e. 9–10-d-old plants) later. In these experiments root hair length was measured at 20 min intervals for a period of 5 h.

Statistical analysis

Data were analysed by ANOVA (Systat Inc., Wilkinson *et al*., 1992). The mean values presented in the root hair growth and pH studies represent at least five replicates, with one root hair being one replicate, from a total of at least three different plants. The root hair growth rate was calculated by differentiation of total root hair length as a function of time, using running quadratic polynomials in 3-point frames (Erickson, 1976). For the analysis of ion gradient studies, each experiment was repeated twice (four plants per treatment, two hairs per plant. Repeated measures analysis was used, using Ca^{2+} and Na^+ and their interaction as the 'between subject' variables. The means from the 'short-term' Ca^{2+} time-course experiment represent data from five different root hairs on five different plants, and were analysed by repeated measures analysis, using time as the 'within subject' variable and Ca^{2+} and Na as the 'between subject' variables.

Results

Cytoplasmic pH is uniform and not affected by NaCl treatment

A representative image of the pH distribution of a root hair is shown in Fig. 1. The pH of the apical 12 µm of cytoplasm was 7.20±0.06, a value consistent with other reports of root hair pH (Katsuhara *et al*., 1997; Bates, 1998). NaCl stress did not significantly affect cytosolic pH in the root hair tip either during 'short term' exposure (for up to 20 min, Fig. 1) or 'long term' exposure of 2–6 d of continuous NaCl treatment (data not shown). The stability of the pH upon NaCl treatment was observed with measurements made with both BCECF-AM loaded root hairs and hairs which were microinjected with BCECF-dextran, suggesting that NaCl did not cause any redistribution of dye or alteration of dye loading, characteristics which could mask any cytoplasmic pH change. These results support conclusions drawn by previous authors that there is no detectable growth-related pH gradient at the root hair apex and root hair cytosolic pH is tightly regulated (Herrmann and Felle, 1995; Bibikova *et al*., 1998; Parton *et al*., 1997).

Rapid effects of NaCl treatment on cytosolic Ca^{2+}

In order to determine any short-term effect of NaCl on the tip-focused, growth-related Ca^{2+} gradient known to exist at the apex of growing root hairs, confocal ratio imaging was used to monitor the dynamics of the gradient as NaCl stress was applied. Figure 2A shows that the Ca^{2+} gradient was detectable in growing root hairs and remained at a level consistent with that reported for actively growing root hairs (Wymer *et al*., 1997) for at least 20 min after application of up to 90 mM NaCl. The numerical results of this experiment are shown in Fig. 3. Ratiometric measurements of cytosolic Ca^{2+} were made every 5 min for 20 min, at the indicated distances from the apex. As shown in Fig. 3, 20 min of exposure to 90 mM NaCl did not alter Ca^{2+} concentrations in the apex. Thus, it is unlikely that the lack of detectable changes in Ca^{2+} were due to NaCl affecting partitioning of dye in a stress-dependent manner as the dextran-conjugated dyes are thought to remain in the cytosol.

In order to test for potential rapid signal-related elevations in Ca^{2+} induced by NaCl treatment, occurring too rapidly to be imaged, Ca^{2+} was monitored towards the apex of the root hair, approximately 10 µm from the very apex. This point was chosen as being where the high Ca^{2+} levels of the tip-focused Ca^{2+} gradient had been reduced to

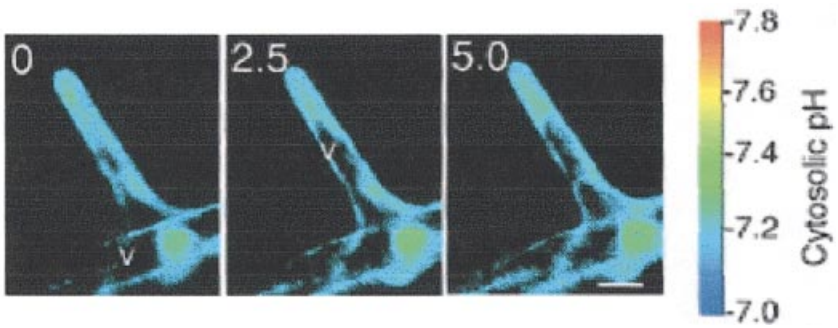

Fig. 1. Cytoplasmic pH within a root hair of *A. thaliana* which has been microinjected with the dual excitation fluorescent pH indicator BCECF-dextran and monitored by confocal ratio imaging. Representative ratio images (n=7) of the effect of 60 mM NaCl added at 0 time. Numbers=minutes after NaCl addition. Scale bar represents 10 µm. The fluorescence signal has been pseudo-colour coded according to the inset scale. V, vacuole.

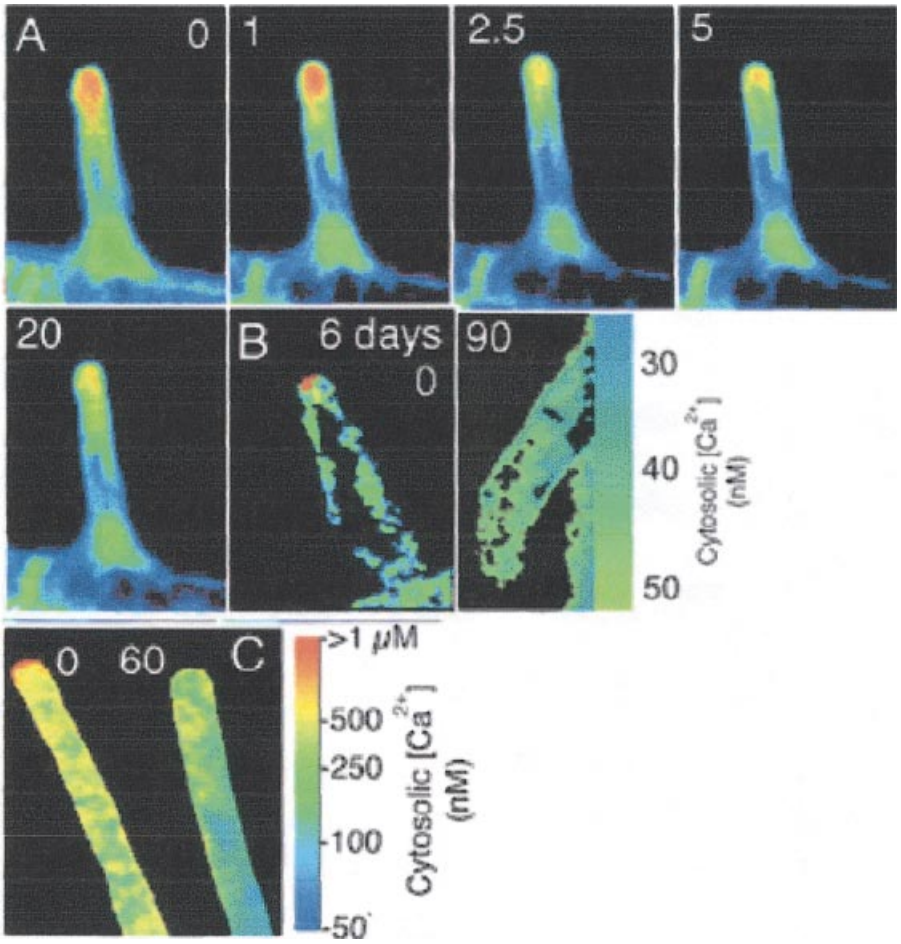

Fig. 2. Cytoplasmic Ca^{2+} within a root hair of *A. thaliana* which has been microinjected with the dual emission fluorescent Ca indicator Indo-1-dextran and monitored by confocal ratio imaging. (a) Representative ratio images (n=9) of the effect of 60 mM NaCl added at 0 time. Numbers=minutes after NaCl addition. Scale bar represents 10 μm. The fluorescence signal has been pseudo-colour coded according to the inset scale. (b) Representative ratio image of Indo-1–dextran microinjected root hair viewed by non-confocal ratio imaging. These root hairs are on plants treated with 0 or 90 mM NaCl. Note elevated Ca^{2+} at very apex of the hair in the control which is lacking in the 90 mM treatment. The scale bar for the 90 mM treatment is separate because cells of this treatment have very low amounts of cytosolic Ca^{2+}. (c) Representative ratio images (non-confocal) of root hairs loaded with Indo-1 by acid loading. The root hairs are from plants grown in 2 mM Ca^{2+} and then for 6 d in medium with 2 mM Ca^{2+} supplemented with or without 60 mM NaCl (numbers represent NaCl concentration). Notice sharp gradient at the tip in the 0 mM NaCl treatment which is reduced in the 60 mM treatment.

basal levels (approximately 50–100 nm), and so elevations in Ca^{2+} might be detectable. The timing was chosen (the first 2 min), because this is the time period in which previously observed Ca^{2+} spikes in response to NaCl treatment had been reported (Lynch *et al*., 1989; Kiegle *et al*., 2000). Measurements were made every 0.5 s using a photometer. Large transient increases in Ca^{2+} were not obvious in this region during the first 2 min following exposure to 0, 30, 60 or 90 mM NaCl and this stable Ca^{2+} level was unaffected by changes in extracellular Ca^{2+} level from 2 to 5 mM (Fig. 4). Although there were statistically significant alterations in cytosolic Ca^{2+} concentration upon salt treatment of root hairs grown in medium containing 0.5 mM Ca^{2+}, the very small absolute magnitude of the changes detected (±10 nM) suggests that, although statistically significant, such alterations may not be linked to biologically relevant responses which are thought to require Ca^{2+} changes of one or two orders of magnitude larger amplitude (Hepler and Wayne, 1985). There is some difference in the magnitude of cytosolic changes in the controls between Figs 3 and 4, most likely due to the difference in instrumentation. The results in Fig. 3 were obtained on a confocal microscope, whereas those in Fig. 4 were obtained on a photometer on a non-confocal microscope which has different optical properties (particularly emission bandpass). However, both of the measurements are within what would be expected for the portion of the cytosol which is not in the gradient region.

Effects of long-term NaCl treatment on Ca^{2+} gradients and root hair extension

To verify that the presence of NaCl did not affect measurements made with acid-loaded Indo-1, root hairs of plants grown in 0.5 mM Ca^{2+} and 90 mM NaCl for 6 d

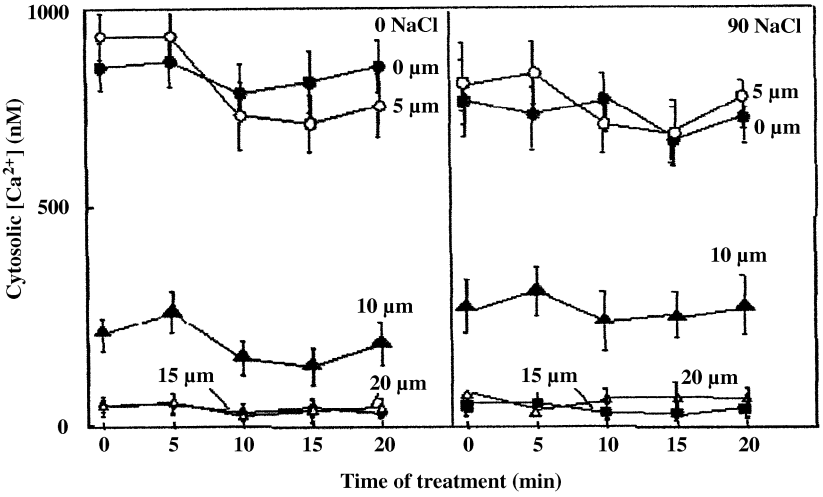

Fig. 3. Average cytoplasmic Ca^{2+} levels measured from root hairs acid loaded with Indo-1 and treated with 0 or 90 mM NaCl. Average cytosolic Ca^{2+} levels were measured in a 5×5 µm region at the indicated distances from the apex of the hair from confocal ratio images from at least 17 individual hairs from >5 separate plants per treatment. Values represent mean ±SEM.

(the most extreme treatment used) were microinjected with dextran-conjugated Indo-1. As shown in Fig. 2B, a sharp Ca^{2+} gradient at the apex is visible in the control and the root hair exposed to 90 mM NaCl has very little Ca^{2+} at the apex. An acid-loaded root hair of a plant grown for 6 d in 60 mM NaCl is shown in Fig. 2C. The NaCl-treated root hair lacks a gradient in this image as well, and the root hair is not particularly stunted. Images of the Ca distribution of a root hair treated with 60 mM NaCl for 6 d and an untreated root hair are shown in Fig. 2C. As expected, the growing, untreated root hair has a high concentration of Ca^{2+} focused on the cytoplasm in the first few microns from the tip. This tip-focused gradient is not present in the NaCl-treated root hair. NaCl sharply reduced the apical concentration of cytosolic Ca^{2+} in root hairs of plants grown with 0.5 or 2.0 mM Ca^{2+}, at either 2 d or 6 d after salinization, and a lesser reduction occurred in the 5.0 mM Ca^{2+} treatment (Figs 5, 6). Root hairs of plants grown with 5.0 mM Ca^{2+} maintained elevated cytosolic Ca^{2+} at the apex despite the extended NaCl treatment. After 2 d of salinization, the extracellular Ca^{2+} level had a statistically greater effect than Na^+ (F=10.12>F=3.12) upon the concentration gradient than did Na^+, and there was no significant effect, based on ANOVA, from the interaction of Ca^{2+} and Na^+. After 6 d of salinization, however, exogenous Na^+ had a statistically greater effect (F=10.11>F=4.40) upon the Ca^{2+} gradient than exogenous Ca^{2+}, and there now was a significant interaction between Ca^{2+} and Na^+ (F=2.34, P <0.05).

The period of stationary growth rate of root hairs in all of the treatments was similar (Figs 7, 9) and in all treatments the maximum extension rate (obtained via numerical differentiation of the data in Figs 7 and 9) of the control treatment was in the middle of the growth period (Figs 8, 10). As NaCl was increased, the growth rate decreased in each Ca^{2+} treatment. Across Ca^{2+} treatments, elevating Ca^{2+} was beneficial for maintaining root hair growth.

External Ca^{2+}, root hair extension rate, and external Na^+ were correlated with the tip-focused Ca^{2+} gradient, as shown in Tables 1 and 2. Although external Ca^{2+} was not significantly correlated with root hair extension rate, it did have a significant effect upon the peak of the Ca^{2+} gradient. The control extension rates were similar in the 0.5 mM Ca^{2+} and 2.0 mM Ca^{2+} treatments, although both were below the rate of the controls in 5.0 mM Ca^{2+}. However, the root hairs grown in 0.5 mM Ca^{2+} were more inhibited by NaCl than those grown in 2.0 mM Ca^{2+}. External Ca^{2+} had a significant effect on the maximum amount of apical Ca^{2+}. The more Ca^{2+} the root hairs received, the more Ca^{2+} was at the apex.

These findings are quantified in the correlation matrix (table 2). External Ca^{2+} has a low correlation with hair extension rate either 2 d or 6 d after application of NaCl, but has a significant correlation with cytosolic Ca^{2+} on both days. Likewise, cytosolic Ca^{2+} is correlated with extension rate on both days. External Na^+ is significantly correlated with cytosolic Ca^{2+} 2 d after application, and the correlation has a low P-value (0.05125) on the sixth day. External Na^+ is highly significantly correlated (P <0.001) with extension rates on both days.

Discussion

The results indicate that salinity stress inhibits root hair growth and cytosolic Ca^{2+} at the apex, although not rapidly. The deleterious effects of salinity stress on root hair growth could be partially reversed by supplemental

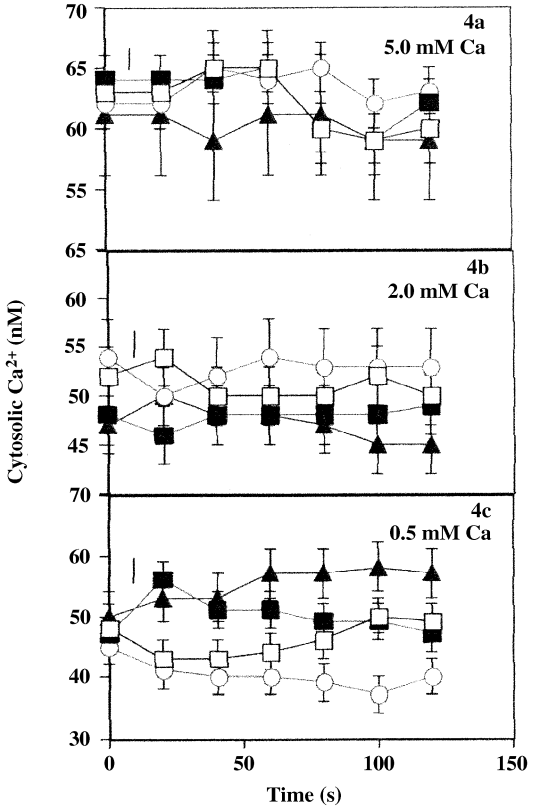

Fig. 4. Cytosolic Ca^{2+} measurements in *A. thaliana* root hairs monitored for 2 min following application of 0 (open squares), 30 (filled squares), 60 (open circles), or 90 mM NaCl (filled triangles) to plants grown in 5.0 mM Ca^{2+} (a), 2.0 mM Ca^{2+} (b), or 0.5 mM Ca^{2+} (c). Measurements taken at intervals of 0.5 s, points on graph represent calculated mean at 15 s intervals. Sodium chloride applied 10 s (indicated by line) after start of measurement. Calcium and the interaction of Ca^{2+} with NaCl had significant effects on cytosolic Ca^{2+} activity (F=80.71, P <<0.0001 and F=7.86, P <0.001, respectively). Within the 0.5 mM Ca^{2+} treatment, NaCl had a significant effect on cytosolic Ca^{2+} activity (F=12.96, P=0.001). Each mean represents measurements from one hair taken from each of five plants. Error bars represent SEM.

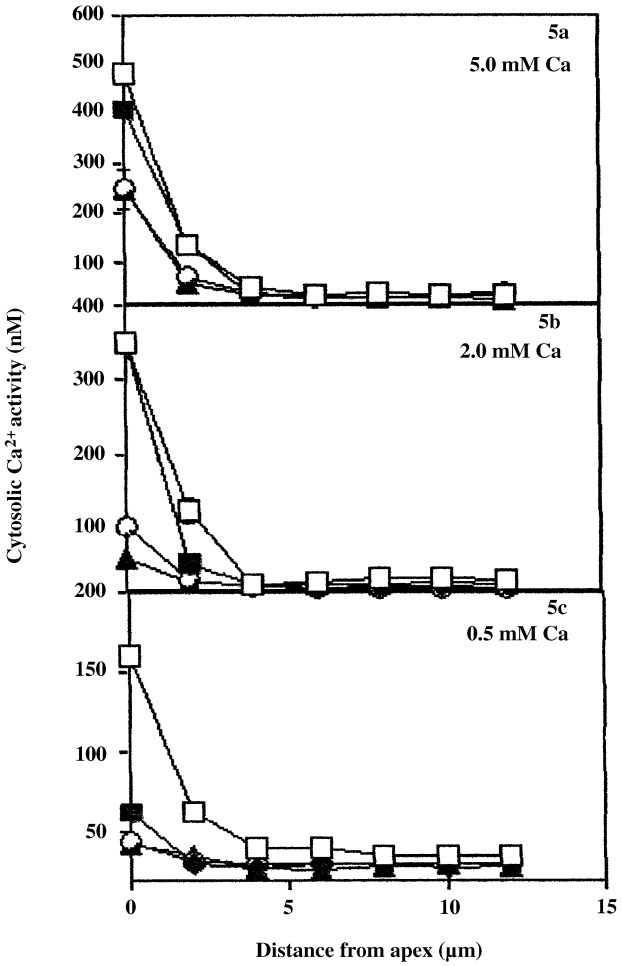

Fig. 5. Ca^{2+} gradients present in root hairs of *A. thaliana* after plants are exposed to NaCl stress for 2 days (0 mM, open squares; 30 mM, closed squares; 60 mM, open circles; 90 mM, filled triangles). Calcium and Na and their interaction had significant effects on cytosolic Ca^{2+} activity (F=10.12, P <0.001; F=3.12, P <0.05), whereas there was no significant interaction between them. Error bars represent SEM, each mean represents data from a total of five root hairs from three different plants.

extracellular Ca^{2+} (Figs 6–9). Schiefelbein *et al.* (1992) reported that at the tip of root hairs of *A. thaliana*, there is Ca^{2+} influx which does not exist along the sides, and this has also been reported for root hairs of *Sinapis alba* (Herrmann and Felle, 1995). The tip-focused Ca^{2+} gradient is known to be important for root hair tip growth (Wymer *et al.*, 1997; Bibikova *et al.*, 1997). Thus, in Bibikova *et al.* (1997), it was reported that when an artificial Ca gradient is generated in the root hair, the root hair grew in the new direction for 15 min, but then returned to the original angle of growth. The Ca^{2+} gradients observed were highly localized, falling within the apical 10 μm. This is consistent with previously reported apical Ca^{2+} gradients (Wymer *et al.*, 1997; de Ruijter *et al.*, 1998). In Wymer *et al.* (1997), the authors demonstrated a correlation between the steepness of the apical Ca^{2+} gradient and root hair growth. This correlation is present in this study too, within a given external Ca^{2+} concentration. However, there was much less correlation between growth rate and external Ca^{2+} level across all three external Ca^{2+} concentrations. For example, the 0.5 mM Ca^{2+} unsalinized treatment grew at the same rate as the 2.0 mM unsalinized treatment, despite having lower absolute magnitude of the Ca^{2+} gradient. Jones *et al.* (1998) also reported that there was not an absolute correlation between the magnitude of the Ca^{2+} gradient and growth rate in root hairs stressed by Al^{3+} treatment. In this study, root hairs of aluminium (Al)-sensitive and Al-resistant *A. thaliana* plants were inhibited in growth by Al, but did not experience a loss of a Ca^{2+} gradient. This lack of a correlation can be explained by the fact that tip growth is a complicated process, involving actin, the activity of many genes, etc. (Dolan, 2001) which

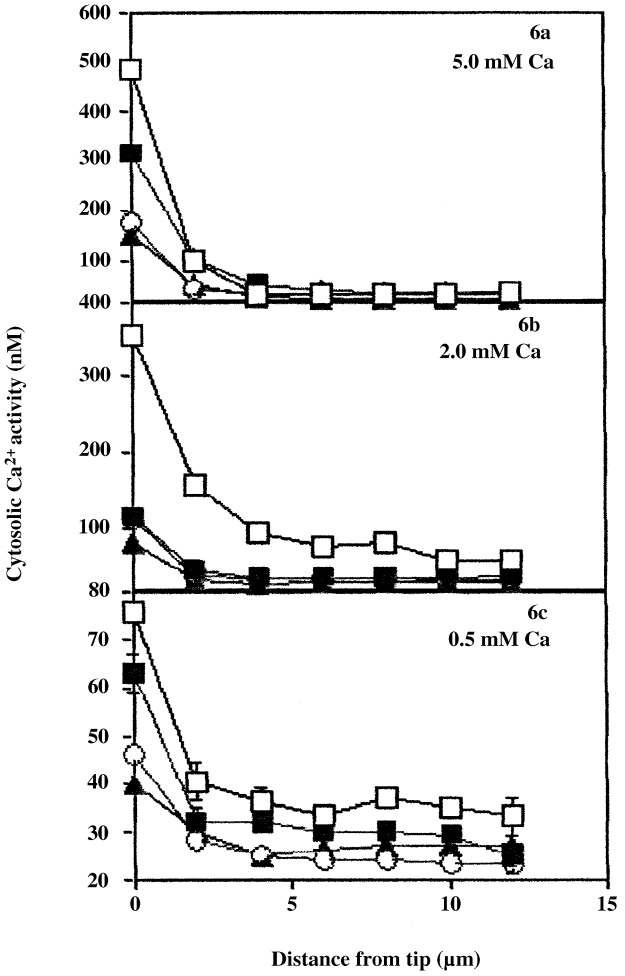

Fig. 6. Ca^{2+} gradients present in root hairs of *A. thaliana* after plants are exposed to NaCl stress for 6 d (0 mM, open squares; 30 mM, filled squares; 60 mM, open circles; 90 mM, filled triangles). Ca^{2+} and Na^{+} and their interaction had significant effects on cytosolic Ca^{2+} activity (F=4.40, P <0.05; F=10.11, P <<0.001; F=2.34, P <0.05). Error bars represent SEM, each mean represents data from a total of five root hairs from three different plants.

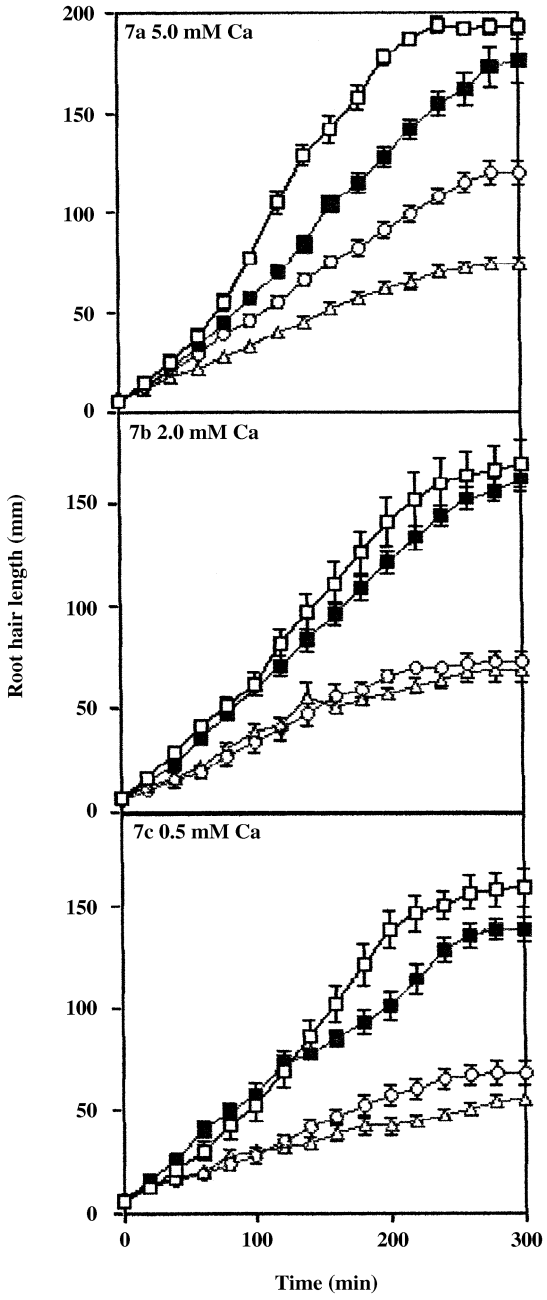

Fig. 7. Extension of root hairs of *A. thaliana* grown in 5.0 (a), 2.0 (b) or 0.5 (c) mM Ca^{2+} and treated with 0 (open squares), 30 (filled squares), 60 (open circles) or 90 mM NaCl (open triangles) for 2 d.

is not fully understood. That Ca^{2+} gradients are associated with growing root hairs is well-documented, but the cytosolic Ca^{2+} gradient formation and root hair growth are still two different processes, and can be affected differentially.

In these experiments, externally applied NaCl did not stimulate a rapid elevation of cytosolic Ca^{2+} in the apex of root hairs. This is in contrast to the responses of other root cells (Lynch *et al*., 1989; Kiegle *et al*., 2000). However, using the aequorin Ca^{2+} measurement system, there appears to be no significant difference in the Ca^{2+} response of wild-type and hairless mutants of *Arabidopsis* to NaCl stress, suggesting that root hairs do not contribute significantly to the NaCl-induced Ca transients which occur in the roots as a whole (H Knight, personal communication 2001).

Cytoplasmic pH was not affected by salinity stress, which is consistent with previous findings (Katsuhara *et al*., 1997). A tip-focused pH gradient was not observed in either the BCECF-AM loaded or BCECF-dextran microinjected root hairs, consistent with other reports of a lack of such a gradient (Herrmann and Felle, 1995; Parton *et al*., 1997; Bibikova *et al*., 1998). Brauer *et al*. (1995) reported that BCECF-AM does penetrate the vacuole in corn root hairs, but could be used simultaneously to determine a

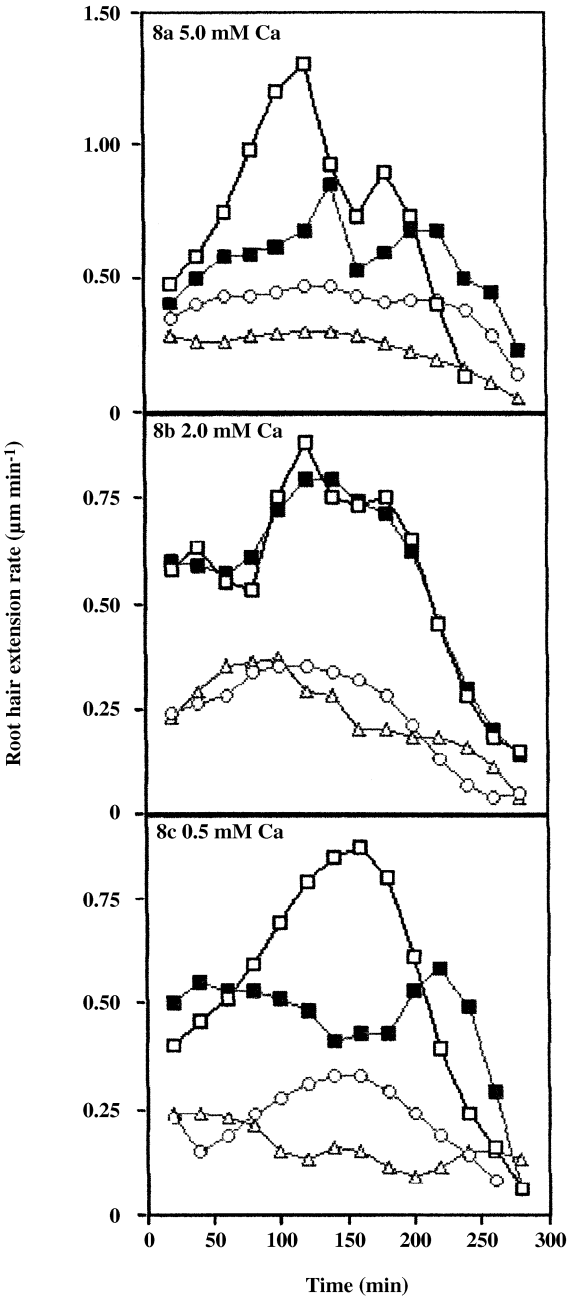

Fig. 8. Extension rate of root hairs of *A. thaliana* plants grown in 5.0 (a), 2.0 (b) or 0.5 (c) mM Ca^{2+} and treated with 0 (open squares), 30 (filled squares), 60 (open circles) or 90 mM NaCl (open triangles) for 6 d. Calcium and Na and their interaction each had highly significant effects (F=38.13, P <0.001; F=228.57, P <<0.0001; F=5.06, P <<0.001). Each mean represents eight hairs taken from three plants, error bars represent SEM. The extension rate has been calculated from the data in Fig. 7 by numerical differentiation and curve smoothing.

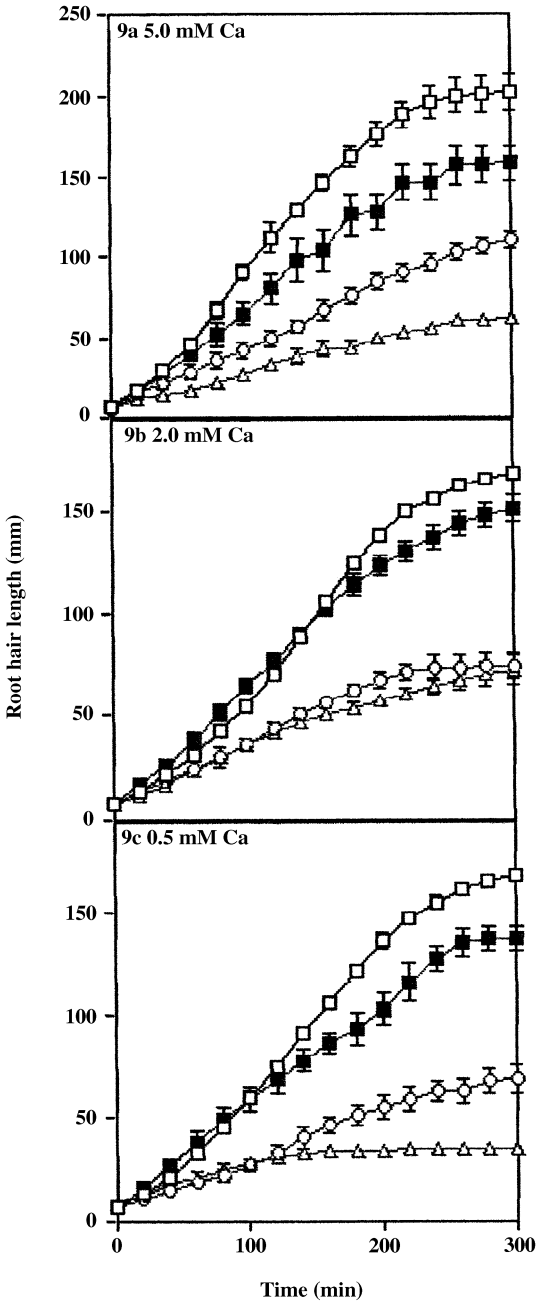

Fig. 9. Growth rate of root hairs of *A. thaliana* plants grown in 5.0 (a), 2.0 (b) or 0.5 (c) mM Ca^{2+} and treated with 0 (open squares), 30 (filled squares), 60 (open circles) or 90 mM NaCl (open triangles) for 6 d.

near-neutral cytosolic pH and acidic vacuolar pH. In this study, pH from BCECF-AM loaded hairs was monitored in the apical 10 μm of the hair, a cytoplasmic-rich region devoid of vacuole, thus the movement of the dye into the vacuole does not seem to interfere with the ability to measure cytosolic pH specifically in this region. It is still possible that BCECF-AM may have partitioned into the abundant secretory vesicles or Golgi found at the apex of the hair and so not be faithfully reporting cytosolic pH. However, such partitioning is not thought to occur with dextran conjugated dyes, and so the observation that microinjected BCECF-dextran gave the same stable, NaCl-insensitive pH signal from the hair apex as seen with BCECF-AM implies both are accurately reporting

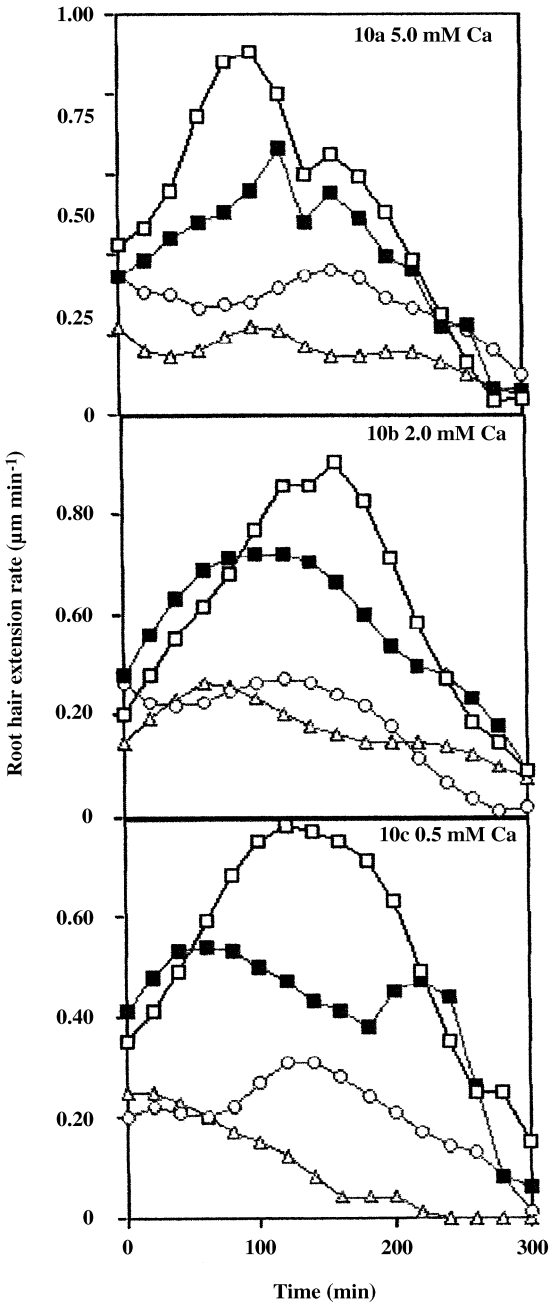

Fig. 10. Extension rate of root hairs of *A. thaliana* plants grown in 5.0 (a), 2.0 (b) or 0.5 (c) mM Ca^{2+} and treated with 0 (open squares), 30 (filled squares), 60 (open circles) or 90 mM NaCl (open triangles) for 6 d. The extension rate has been calculated from the data in Fig. 9 by numerical differentiation and curve smoothing.

that NaCl treatment does not inhibit root hair extension by altering cytosolic pH at the growing tip.

Much recent research has demonstrated the importance of root hairs in nutrient uptake (reviewed in Gilroy and Jones, 2000). There are many mechanisms by which Ca^{2+} could enhance plant cell growth under NaCl stress, such as enhancing K^+/Na^+ selectivity and reducing Na^+ content (from melon plants, Navarro *et al*., 2000) and preventing membrane leakiness (Marschner, 1986). More recently, a series of genes (*sos*1-4, Wu *et al*., 1996; Qiu *et al*., 2002) and *sos*4 (Shi and Zhu, 2002; Shi *et al*., 2002) have been described which could have direct relevance to this research. *sos*1 codes for a Na^+/H^+ antiport, which is required for growth and potassium under salinized conditions (Wu *et al*., 1996). *sos*2 and *sos*3 require Ca^{2+}, and together regulate *sos*1 (Qiu *et al*., 2002). *sos*4 (Shi and Zhu, 2002; Shi *et al*., 2002) codes for pyridoxal kinase, and plants deficient in this gene are hypersensitive to Na^+ ions, and under saline conditions accumulate more Na^+ and less K^+. Interestingly, this mutant also blocks the initiation of root hairs and impairs the tip growth of existing hairs. Another unexplored avenue in the topic of NaCl stress and root hair growth is the importance of membrane polarity. Very and Davies (2000) have reported the presence of a hyperpolarization-activated calcium channel, which they suggest is a positive feedback on root hair growth. Sodium can affect membrane polarity of plant cells (Babourina *et al*., 2000), and by this mechanism it can affect root hair extension and Ca^{2+} uptake.

A broader ramification of this work is a possible connection to the previously described phenomenon that NaCl stress has been shown to be more deleterious to symplastic xylem loading of Ca^{2+} than apoplastic loading of Ca^{2+} into the xylem (Halperin *et al*., 1997; Lynch and Lãuchli, 1985). In the symplastic pathway, Ca^{2+} must enter the endodermal cells. If these cells have depressed cytoplasmic Ca^{2+} when exposed to extended NaCl stress this should affect loading of Ca^{2+} into the xylem. The present data show that extended NaCl stress can disturb cytoplasmic Ca^{2+}, albeit at the tip-focused gradient more than in basal cytoplasm. In the aforementioned study, it was also demonstrated that supplemental Ca could alleviate the NaCl-induced loss of xylem loading of Ca^{2+}. The results in this present study would suggest elevated extracellular Ca^{2+} should help sustain a normal Ca^{2+} homeostasis even under extended NaCl stress. Since reduced Ca^{2+} availability in the growth zone of salinized leaves could be an important component of salt stress in whole plants (Lynch *et al*., 1988), salinity effects on cytosolic Ca^{2+} activity in endodermal cells could be an important mechanism of salinity stress.

In summary, NaCl caused a reduction of root hair extension and a reduction in the magnitude of the cytosolic Ca^{2+} gradient focused to the tip of the hairs. These effects were reduced by supplemental exogenous Ca^{2+}. There were no dramatic shifts of cytoplasmic Ca^{2+} level in the basal region of the gradient and NaCl had no effect on pH, either in the short- or long-term. Root hairs play an important role in water and nutrient acquisition, and are among the first cells to come into contact with salinized soil water. Thus, the effects reported here on root hair growth and Ca^{2+} gradients have the potential to contribute

Table 1. *The effect of NaCl stress for 2 or 6 d on maximum extension rate of root hairs of* Arabidopsis thaliana *and cytosolic* Ca^{2+} *at the hair apex* (Ca^{2+}); n=*5*±*SEM*

External concentrationn (mM)		2 d		6 d	
Ca^{2+}	Na^{+}	Rate (μm min^{-1})	Ca^{2+} (nM)	Rate (μm min^{-1})	Ca^{2+} (nM)
5.0	0	1.30	481 (11)	1.13	486 (15)
5.0	30	0.85	408 (12)	0.83	316 (7)
5.0	60	0.47	249 (13)	0.45	177 (12)
5.0	90	0.30	249 (40)	0.28	150 (8)
2.0	0	0.88	348 (13)	0.90	357 (7)
2.0	30	0.79	348 (13)	0.65	116 (10)
2.0	60	0.34	106 (7)	0.33	113 (9)
2.0	90	0.28	63 (1)	0.35	83 (8)
0.5	0	0.87	159 (2)	0.79	76 (2)
0.5	30	0.43	62 (4)	0.60	63 (2)
0.5	60	0.33	43 (4)	0.23	46 (4)
0.5	90	0.16	42 (4)	0.28	40 (1)

Table 2. *Pearson correlation matrix (upper table) and matrix of probabilities (lower table) of the means presented in Table 1*

Bartlett chi-square statistic is 77.311, df=15.

	External Ca^{2+}	Cytosolic Ca^{2+} (2 d)	Hair extension rate (2 d)	Cytosolic Ca^{2+} (6 d)	Hair extension rate (6 d)	External Na^{+}
External Ca^{2+}	1.00					
Cytosolic Ca^{2+} (2 d)	0.72	1.00				
Hair extension rate (2 d)	0.35	0.83	1.00			
Cytosolic Ca^{2+} (6 d)	0.67	0.88	0.80	1.00		
Hair extension rate (6 d)	0.29	0.78	0.96	0.80	1.00	
External Na^{+}	0.00	–0.58	–0.89	–0.57	–0.90	1.00
External Ca^{2+}	0					
Cytosolic Ca^{2+} (2 d)	0.00776	0				
Hair extension rate (2 d)	0.26868	0.00076	0			
Cytosolic Ca^{2+} (6 d)	0.01802	0.00016	0.00188	0		
Hair extension rate (6 d)	0.36654	0.00295	0.00000	0.00197	0	
External Na^{+}	1.00000	0.04783	0.00012	0.05125	0.00006	0

significantly to nutrient deficiency symptoms seen in salt-stressed plants.

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