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RESEARCH PAPER

Effects of salinity on cytosolic Na^+ and K^+ in root hairs of *Arabidopsis thaliana*: *in vivo* measurements using the fluorescent dyes SBFI and PBFI

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Abstract

Toxicity from sodium accumulation is an important aspect of salinity stress that has been well studied at the organ and tissue level. However, the effects of salinity on sodium accumulation in the cytosol, where much of the sodium toxicity is thought to occur, are poorly understood due to the difficulty of direct non-invasive measurements of ion activities in living cells. The Na^+-sensing fluorescent probe sodium-binding benzofuran isophthalate (SBFI) and the K^+-sensing fluorescent probe potassium-binding benzofuran isophthalate (PBFI) were used to quantify Na^+ and K^+ activity in living root hairs under salinity stress. The effects of exposure of *Arabidopsis thaliana* roots to 0, 30, 60 or 90 mM NaCl were observed during the 20 min immediately following salinization and also after 2 d of salinization, in plants supplied with 0.5, 2.0 or 5.0 mM Ca. SBFI and PBFI fluorescence was confined primarily to the cytoplasm, with very little signal from the vacuole. Sodium affected the quantification of K^+ by PBFI, thus limiting the usefulness of this dye. Root hairs exposed to NaCl accumulated from 30–60 mM Na^+ within the first 5 min of salinization in 0.5 and 2.0 mM Ca^{2+}, and up to 15 mM Na^+ in the 5.0 mM Ca^{2+} treatment. Two days of salinization did not increase cytosolic Na^+ concentrations beyond the values observed after 20 min of salinization. Cytosolic activities roughly corresponded with elemental analysis of combined dry matter fractions from whole plants. We conclude that SBFI and, to a lesser extent, PBFI are useful tools for quantifying the dynamics of ion activities in the cytosols of living plant cells.

Key words: *Arabidopsis*, cytosolic K, cytosolic Na, fluorescent dyes, root hairs, salinity.

Introduction

Sodium chloride stress disturbs the ionic balance of plant cells and organs; it can result in a loss of Ca^{2+} and K^+ from plant cells (Lynch *et al*., 1988; Tarakcioglu and Inal, 2002) and a reduction of K^+-influx in roots as well (Cramer *et al*., 1987; Silberbush and Ben-Asher, 1987). Sodium can enter plant cells through several types of channels: non-selective cation channels (Demidchik and Tester, 2002), voltage-independent channels (Maathuis and Sanders, 2001), and low-affinity inward-rectifying K^+ channels (Maathuis and Sanders, 1995). One of the characteristics of salt-tolerant plant cells is an ability to retain K^+ (Trivedi *et al*., 1991). Supplemental calcium can alleviate salinity stress by various mechanisms, for example, by aiding root elongation and retention of intracellular K^+ (Nakamura *et al*., 1990), enhancing K^+/Na^+ selectivity, reducing Na^+ influx (Cramer *et al*., 1987), reducing the influx of Na^+ through the low-affinity cation channel protein LCT1 (Amtmann *et al*., 2001) and indirectly regulating the Na^+/H^+ antiport, which is responsible for Na^+ efflux from the cell, via the calcium sensor coded by *sos*3 (Qiu *et al*., 2002), and the transformation of *A. thaliana* with a vacuolar Na^+/H^+ antiport from yeast enabled the plants to grow in medium containing as much as 200 mM NaCl (Apse *et al*., 1999).

Because sodium chloride stress is such an important topic, quantifying Na^+ activity in plant cells is very important. Recent advances in microelectrodes (Carden *et al*., 2001), patch-clamping (Demidchik and Tester, 2002), fluorescent dyes (Mühling and Läuchli, 2002; Mühling and Sattelmacher, 1997; Lindberg, 1995; Felle and Hepler, 1997), and NMR technologies such as ^{23}Na-NMR (Olt *et al*., 2000) allow researchers to measure ion activities in living plant cells. Fluorescent dyes have the advantage of not being mechanically invasive and they allow for real-time measurements of cellular events with high spatial resolution (Tsien and Poenie, 1986; Felle and

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Hepler, 1997). Even though the dyes are not mechanically invasive, they are chemically invasive and care must be taken that the results are not artefacts of dye loading and subcellular compartmentalization, as can happen with BCECF-AM (Brauer *et al*. 1995) and PBFI-AM (Lindberg, 1995). ^{23}Na-NMR is not invasive, and has a high degree of spatial resolution (Gruwel *et al*., 2001), which allows researchers to make maps of ion concentrations, including vacuolar measurements. However, two difficulties are image acquisition time in the time frame of minutes (Olt *et al*., 2000), and a narrow set of measurable ions. Microelectrodes allow for high temporal resolution, and have been used to measure Na^+ activity in root cells (Carden *et al*., 2001, 2003), K^+ activity equalling 235 mM in the vacuole of barley root cells grown under K^+-replete conditions (Cuin *et al*., 1999); vibrating microelectrodes may be used to monitor Ca^{2+} fluxes at the epidermis of roots (Kochian *et al*., 1992; Halperin *et al*., 1997; Shabala and Newman, 1998). However, microelectrodes are spatially limited; without a pH sensor Carden *et al*. (2001) were not able to distinguish the difference between a vacuolar and a cytosolic signal, and recommended the use of triple-barrelled microelectrodes equipped with a pH sensor. Felle and Hepler (1997) combined measurements made by the Ca^{2+}-sensing dye Fura-2 with measurements made by Ca^{2+}-sensing microelectrodes to describe Ca^{2+} dynamics in *Sinapis alba* root hairs; such an approach allowed them to combine the strengths of both techniques.

Surprisingly, there have been few attempts to use fluorescent dyes to investigate ionic concentrations in salt-affected plant cells. Indo-1 has been employed to measure Ca^{2+} gradients in NaCl-stressed root hairs of *A. thaliana*, and sodium-binding benzofuran isophthalate (SBFI) has been used to measure Na^+ in the apoplast of cotton leaves (Mühling and Läuchli, 2002). Lindberg (1995), Lindberg and Strid (1997), and Mühling and Sattelmacher (1997) have demonstrated that potassium-binding benzofuran isophthalate (PBFI) can be used to quantify K^+ in plant cells and in the apoplast. The aim of this study was to investigate whether SBFI could be used to measure Na^+ within the cytosol of plant cells. If so, then it would make a valuable tool to complement measurements made with microelectrodes since it causes no mechanical damage. For example, one could envision an experimental set-up in which a vibrating microelectrode is measuring fluxes from the apex of a root hair which has been loaded with SBFI. That way, Na^+ flux and activity within the cytosol could be measured within the same time frame.

In this report, the results are presented of the measurements of cytosolic Na^+ and K^+ in root hairs of *A. thaliana*. Three experiments are discussed: measurement of K^+ in the cytosol of root hairs grown at different Ca^{2+} concentrations, measurement of Na^+ in the cytosol of root hairs for a period of 20 min after NaCl application, and measurement of the accumulation of Na^+ in root hairs of plants that had been grown for several days in medium supplemented with NaCl and also at different levels of Ca^{2+}. It is hypothesized that supplemental Ca^{2+} will decrease intracellular Na accumulation, based on previous reports that an increase in Ca^{2+} supports Na^+ exclusion from plant tissues (Cramer *et al*., 1987), and on reports referred to above which demonstrate that Ca^{2+} hinders the entry of Na^+ into plant cells.

Materials and methods

Seedling material and growth conditions

Seeds of *A. thaliana* L. (ecotype Columbia) were surface-sterilized in 95% ethanol for 5 min, followed by 10% NaOCl for 5 min, and then were rinsed in sterilized water. They were planted onto sterilized solid medium consisting of 2% sucrose, 0.25% Phytagel (Sigma), 0.55 mM *myo*-inositol, 2.5 mM 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 was adjusted according to the experimental treatment.

Two millilitres of the solid medium itself was spread onto a 24×60 mm coverslip (two coverslips per 15×100 mm round Petri dish; material from Fisher Scientific), and allowed to harden in a sterile transfer hood for 1 h. After the seeds were sterilized, one seed was placed into the medium on each coverslip. The seeds were germinated in the dark for 1 d, and were placed into constant light (40 μmol m^{-2} s^{-1} photosynthetically active radiation) for the remainder of the experiment. After 3 d, the roots were 1 cm long, and this was the standard length for the start of all the experiments. During the entire period of growth, the Petri dishes were angled at 30° to encourage root growth towards the bottom of the gel. For observation on the microscope, the slab of gel containing the seedling was transferred to another coverslip, which had been glued into a Petri dish (to prevent leakage onto the microscope).

Dye loading

SBFI and PBFI tetraammonium salts (Teflabs, Inc.) were dissolved to 20 μM in TRIS buffer pH 8.2. The salt form was used so as to avoid the potential difficulties of the dye ester partitioning into the vacuole (as noted by Lindberg, 1995). Twenty microlitres of 20 μM SBFI or PBFI were pipetted onto the gel in which the seedling was growing, directly over the root tip, and the seedlings were placed for 1 h in the dark. Under these growth conditions, root hairs of *A. thaliana* grow at approximately 1 μm min^{-1} (Halperin *et al*., 2003). After 1 h exposure, root hair growth was the same, cytoplasmic streaming was active, and the cells were highly vacuolated. These were the same criteria which Felle and Hepler (1997) used to judge the suitability of root hairs for experiments.

Microscope and imaging equipment

The fluorescence studies were carried out with a Nikon Diaphot inverted microscope (Garden City, NJ) with a 40× oil immersion objective lens (Nikon, Japan, numerical aperture=1.30). For SBFI and PBFI measurements, light of 350 nm ±5 nm and 385±7 nm were isolated from a 75 W xenon arc lamp by two scanning monochromators (Photon Technology International, South Brunswick, NJ). These wavelengths were selected because they represent the maximum and minimum points of the excitation spectrum for the dye and are commonly used wavelengths for both dyes (Minta and Tsien, 1989). Excitation and emission wavelengths were separated by a 400 nm dichroic mirror equipped with a 510± 25 nm emission filter. For capturing images, fluorescence emission

intensity was increased by a Hamamatsu Photonics C2400 Intensifier (Hamamatsu, Japan). Fluorescence and bright field images were collected with a Hamamatsu Photonics XC-77 CCD camera (Hamamatsu, Japan). Uniblitz excitation shutters and CCD camera were controlled by Metamorph Software (Universal Imaging Corporation, West Chester, PA).

Ratio image analysis

Metamorph version 3.0 imaging software (Universal Imaging Company, West Chester, PA) was used for ratio analysis of raw data image pairs. Setting the intensifier to a setting of 7.0, and setting the analogue contrast to a white level of 51 and a black level of 27 eliminated autofluorescence from the root hair, but not from the main body of the root. To make a ratio image, one 350 nm image and one 385 nm image was captured, and a linescan (5 pixel width) was made at the tip. The grey values from each image were then noted in a notebook and ratioed directly.

Standard curves

The roots were loaded with SBFI or PBFI and after 1 h were treated with Na^+ or K^+ standards and 2 μM gramicidin. Monensin, which mediates Na^+-H^+ exchange (Harootunian *et al*., 1989) was originally tried, but yielded poor results. Gramicidin was used because it forms transmembrane pores through which cations can pass, but it is not selective for Na^+ or K^+ (Harootunian *et al*., 1989). For K^+, there is an inherent difficulty in that the cell has K^+ in it, and it is impossible to buffer the K^+ to a value of zero. Therefore, an *in vitro* curve was performed as well, using 20 μM SBFI or PBFI. Due to the lack of effective selectivity of PBFI for K^+ over Na^+, standard curves of K^+ were made along with 0, 10, 20 or 30 mM Na^+ to determine the effect of Na^+ on quantification of K^+ with PBFI.

Short-term intracellular ion concentration experiments

Seedlings were grown in media containing 0.5, 2.0 or 5.0 mM Ca^{2+}. For short-term Na^+ studies, roots were placed on the microscope and the 350 nm and 385 nm images were obtained and measured. Five microlitres of 0, 30, 60 or 90 mM NaCl were added to the root, and images were taken once a minute for 5 min, and then at 10 and 20 min after salinization.

Long-term intracellular ion concentration experiments

Seedlings were grown in media containing 0.5, 2.0 or 5.0 mM $CaSO_4$. When the roots were 1 cm long (3–4-d-old), medium containing NaCl at twice the final desired concentration was added to an equal amount of gel containing the seedling, which resulted in the following concentrations: 0, 30, 60 or 90 mM NaCl. The 0–60 mM concentrations were confirmed by staining the medium with SBFI. Root hairs from the main root were analysed for intracellular Na^+ 2 d (5–6-d-old) or 6 d (9–10-d-old) later.

Long-term intracellular ion concentration experiments using entire seedlings

Over 100 seedlings per Petri dish (considered one replicate) were grown for 6 d on media containing 0.5, 2.0 or 5.0 mM Ca^{2+} and when they were 6-d-old, an equal volume of liquid medium supplemented with 0, 60, 120 or 180 mM NaCl (to result in final concentrations equal to the above-described experiments) was applied and the seedlings were grown in the saline conditions for 2 d, to equal the time length in the above-described experiment in tissue culture medium and NaCl added as described above. After 2 d, the seedlings were removed from the gel. The shoots, which were erect and nearly perpendicular to the medium, were removed first to prevent any possible contamination by contact with the gel medium and the ions therein and then the roots were removed from the medium. Shoots and roots were rinsed several times in distilled water to remove external ions, and the shoots and roots were separated, dried at 70 °C for 48 h, weighed to determine dry mass, and analysed separately. For analysis, shoot or root tissue was ashed with three drops of 5% H_2SO_4 at 550 °C for 3 h and resuspended in 2 ml of 5 mM HCl. They were then vortexed for 10–15 s each and heated to near boiling (approximately 85–90 °C) for 15–20 min, vortexed again and then vacuum filtered through 0.45 μm pore-size MAGNA plain nylon membrane filter paper (Osmonics MSI, Minnetonka, MN USA). The samples were then diluted 25-fold in 1 mM HCl for ion chromatography using a Dionex DX 500 Ion Chromatograph.

Statistical analysis

Data were analysed by ANOVA using Systat (Systat Software Inc., Richmond, CA). All means represent eight replicates (two root hairs/seedling from at least four different seedlings) and the data were analysed using multivariate ANOVA. Repeated measures analysis was used for the short-term Na^+ uptake studies, using time as the 'within subject' variable, and Ca^{2+} and Na^+ as the 'between subject' variables.

Results

Dye distribution and ratio images

The bright-field images of root hairs (Figs 1a, 2a) and images from root hairs that have been loaded with SBFI or PBFI and illuminated with 385 nm light (Figs 1b, 2b) show fluorescence resulting from the dye distribution of SBFI (Fig. 1b) or PBFI (Fig. 2b) colocalized with the cytosol at the apex, and ratiometric images of hairs loaded with SBFI or PBFI are shown in Figs 1c and 2c, and demonstrate that there is sufficient signal present to allow for measurements of Na^+ and K^+, although these dyes are faint when compared with other dyes, such as Indo-1 (Minta and Tsien, 1989). As shown in the bright-field images, the cytosol occupies a small volume within the root hair; the large central vacuole extends almost to the apex (Peterson and Farquhar, 1996; Foreman and Dolan, 2001). The vacuole forms a tube within the root hair which occupies a large portion of its volume, thus most of the cytoplasm is localized at the tip and also appears as discontinuous, wispy strands streaming around the extended portion of the vacuole, which are in constant motion, and this includes the fraction of cytosol in the thin space between the tonoplast and the plasma membrane. Thus, in the vacuolar region small flecks of cytosol which contain dye in the region between the tonoplast and the cell wall are visible. In the SBFI-treated root hair the flecks are visible as small dots along the length, and in the PBFI-treated root hair the dye is more uniformly distributed along the edges. In single-wavelength and ratiometric images, there is little signal from the central vacuolar region. The acidic pH of the vacuole would result in a very low dye signal (Mühling and Läuchli, 2002) because of poor dye solubility (based on the chemistry of the carboxylic acid groups, A Minta, personal communication). The difference in the dye distribution between the SBFI image, which is patchy, and the PBFI image, which is more uniform, may be due to

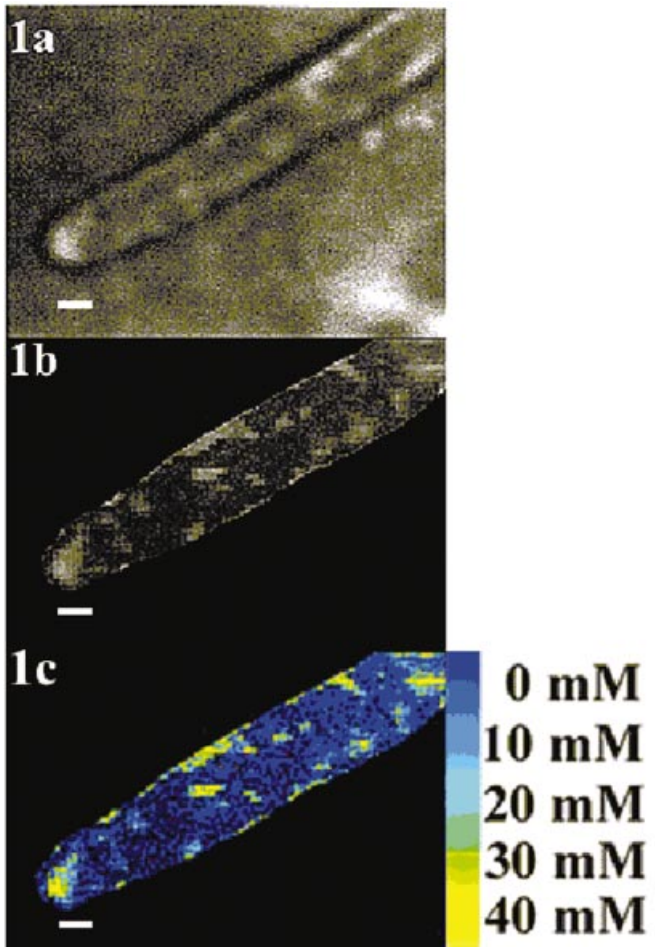

Fig. 1. Bright-field image (a), image of hair illuminated with 385± 7 nm light (b), and image ratioed from images taken at 350±5 nm and 385±7 nm light (c) of root hairs of *A. thaliana* loaded with SBFI. The single wavelength image shown in (b) illustrates the accumulation of dye within the cytosol, which is localized at the tip. The ratio image is used for quantification of Na^+ within the cell. The root hair was observed with a 40× oil immersion lens and an image was captured by CCD camera, the microscope being equipped with a 10× ocular lens. White line equals 10 μm.

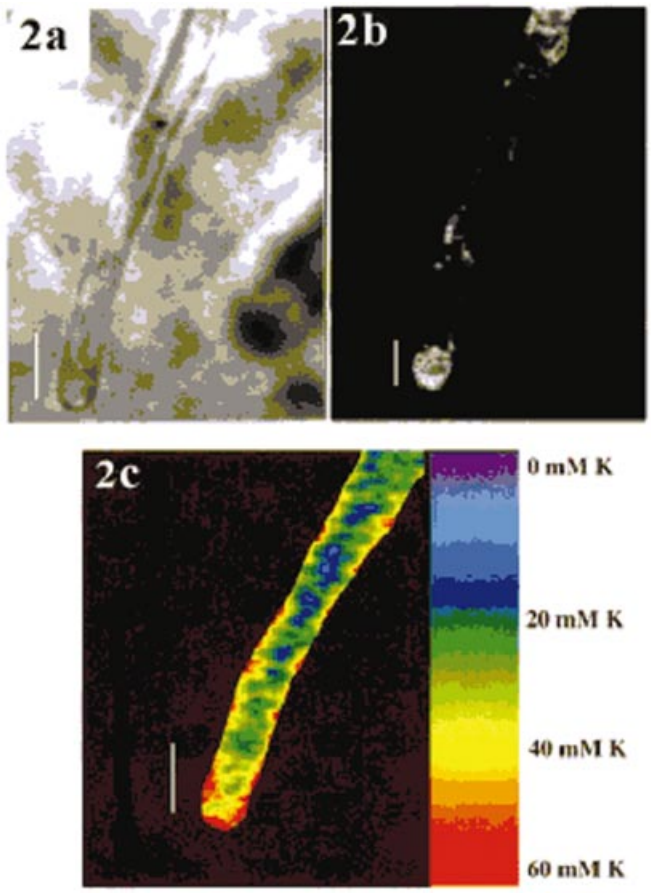

Fig. 2. Bright-field image (a), image of hair illuminated with 385± 7 nm light (b), and image ratioed from images taken at 350±5 nm and 385±7 nm light (c) of root hairs of *A. thaliana* loaded with PBFI. The single wavelength image shown in (b) illustrates the accumulation of dye within the cytosol, which is localized at the tip. The ratio image is used for quantification of K^+ within the cell. The root hair was observed with a 40× oil immersion lens and the image was captured by CCD camera, the microscope being equipped with a 10× ocular lens. White line equals 10 μm.

cytosolic streaming, as cytosol which contains dye moves in and out of the focal plane along the tonoplast. Another factor to be taken into account is the possibility of optical variation resulting from fluorescent signals from focal planes above and below the region of interest, and the curvature of the root hair at the edges. However, because the *in vivo* standard curves are very similar to the *in vitro* standard curves, described below, the effects did not seem to affect the usefulness of the technique to measure cytosolic Na^+ and K^+. These results contrast with those of Lindberg (1995), who reported dye fluorescence in the vacuole without describing the importance of the pH of the vacuole. At this date, dextran conjugates of these dyes are not available, therefore a means of confirming that the results are from a cytosolic signal are not available at this time. The brightness at the base of the root hair results from autofluorescence from the body of the root, which is of such brightness that it cannot be eliminated by adjustments made to eliminate background fluorescence which emanates from the shaft and apex of the growing hair.

Standard curves

SBFI and PBFI were both responsive in the 0–60 mM range, and the *in vivo* curves were very similar to the *in vitro* curves (Figs 3–4). The ratio values for 0–10 mM differed in the two curves for SBFI, the *in vivo* curve going from 0.25–0.80, and the *in vitro* curve ranging from 0.55–0.82. The difference is most likely due to small differences in the optical path when making *in vitro* and *in vivo* curves, since the *in vitro* curves are made with a drop of solution on the microscope, and the *in vivo* curves were made at the apex of the cells. Such an effect is not observed in

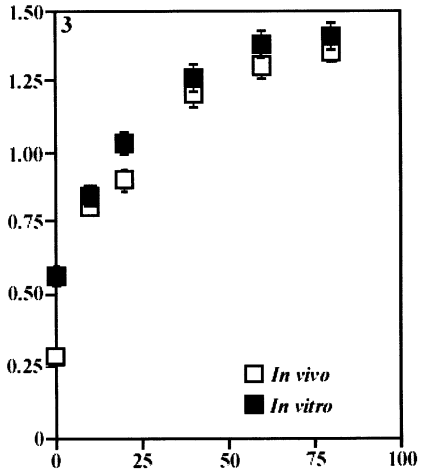

Fig. 3. *In vivo* (a) and *in vitro* (b) standard curves for measuring Na^+ in cells with the ratiometric dye SBFI. For the *in vivo* standard curve, root hairs were loaded with 20 μM dye, and then were treated with an Na^+ solution and 1 μM gramicidin. Sodium had a significant effect on the *in vivo* ratio (F=113.24, P <0.001) and on the *in vitro* ratio (F=94.51, P <0.001). Each mean represents measurements from eight hairs.

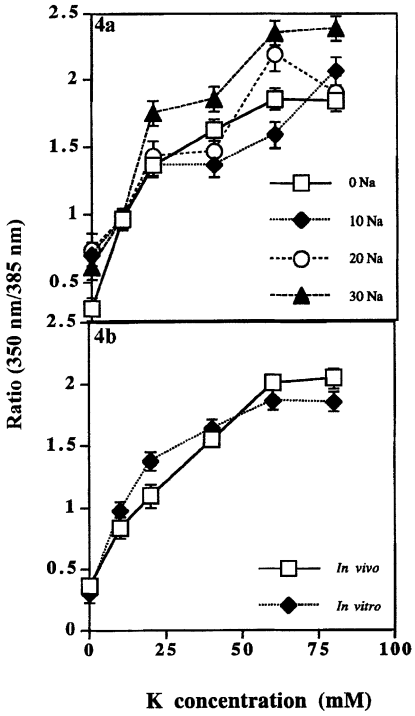

Fig. 4. Standard curves for measuring K^+ in cells with the ratiometric dye PBFI. (a) The effect of increasing Na^+ on the ability to measure K^+ with PBFI, (b) a comparison of the *in vivo* and *in vitro* curves. For the *in vivo* standard curve, root hairs were loaded with 20 μM dye, and then were treated with an Na^+ solution and 1 μM gramicidin. Potassium has a significant effect on the *in vivo* ratio (F=77.77, P <0.001) and on the *in vitro* ratio (F=94.51, P <0.001). Potassium, Na^+, and the interaction have a significant effect on the *in vitro* ratio (F=148.60, P <0.001; F=17.26, P <0.001; F=3.54, P <0.001, respectively). Each mean represents measurements from eight hairs.

measurements made with PBFI. Potassium, in the range to be expected in cytoplasm, does not limit the range of responsiveness of SBFI to Na^+ (Minta and Tsien, 1989). Sodium strongly influenced the response of PBFI to K^+ (Fig. 4a, b), thus limiting the usefulness of this dye under conditions of high NaCl. While PBFI cannot be used to measure small changes (<20 mM) in K^+ concentration, the dye may be useful as an estimator of large changes of K^+ concentration in the presence of Na^+. Possible variation resulting from changing dye concentrations is thought to be minimized by ratioing two images for a measurement (Grynkiewicz *et al*., 1985), hence the reason for the widespread use of ratiometric dyes; Mühling and Läuchli (2002) reported that dye concentration changes from 5–20 μM cause the greatest change in ratio values for a given [Na^+] (from a ratio of 4.5 to 3 for 20 mM NaCl), from 20–100 μM the change was from 3 ratio units to 1 ratio unit. The extremely similar ratio values obtained for the *in vivo* and *in vitro* curves in this study suggest that changing dye concentrations were not a great factor; Mühling and Läuchli (2002) concluded the same for apoplastic measurements. Twenty micromolar was the concentration used in these studies because it gave the brightest possible signal from the root hair and did not have observable effects on root hair growth, and was consistent with the acid-loading concentration of Indo-1, which has been used to measure cytosolic Ca^{2+} (Halperin *et al*., 2003).

Short-term Na uptake

In all of the treatments, most of the Na^+ was taken up within the first 5 min (Fig. 5a–c). In the high Ca^{2+} treatment (5.0 mM Ca^{2+}), very little Na^+ accumulated in the cytosol; the most extreme NaCl treatment resulted in a final cytosolic Na^+ concentration of 10–15 mM (Fig. 5a). At 2.0 mM Ca^{2+}, the 90 mM NaCl treatment resulted in cytosolic accumulation of 60 mM Na^+, but the 30 and 60 mM NaCl treatments resulted in a cytosolic Na^+ concentration of about 20 mM (Fig. 5b). In the low Ca^{2+} treatment (0.5 mM), all root hairs had nearly 60 mM Na^+ in the cytosol (Fig. 5c).

Long-term Na⁺ uptake

Two days after salinization there was an inverse relationship between the amount of Ca^{2+} in the medium and the concentration of Na^+ in the cytosol (Fig. 6). There was also a positive relationship between the concentration of medium NaCl and the cytosolic Na^+ level (Fig. 6). Hairs from seedlings 6 d after salinization could not be measured because the ratios measured were much greater than the values obtained in the standard curve. The standard curve was done on hairs from healthy seedlings, and the ratios saturated at about 1.5, whereas hairs from the day 6 measurements had ratios of 3–5 units. A plausible reason for this is that the root hairs had lost a lot of K^+, which

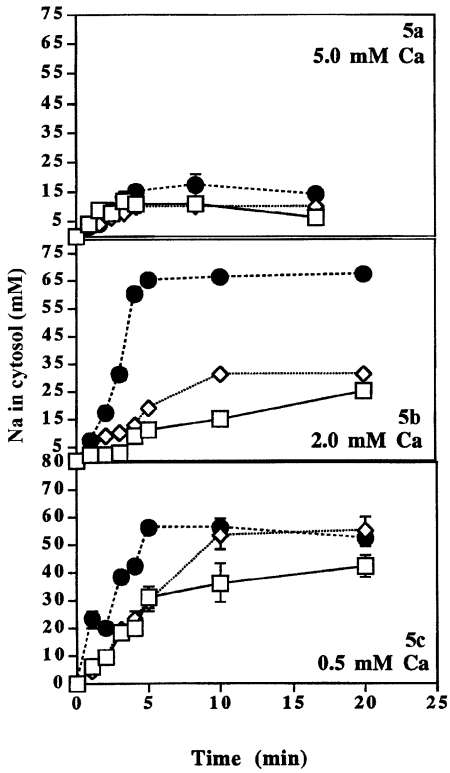

Fig. 5. Uptake of Na^+ into the cytosol of root hairs of *A. thaliana* grown in 5.0 mM Ca^{2+} (a), 2.0 mM Ca^{2+} (b), or 0.5 mM Ca^{2+} (c) when given 30 mM NaCl (squares), 60 mM NaCl (diamonds), or 90 mM NaCl (circles). Sodium and Ca^{2+} and the interaction had significant effects (F=8.06, P <0.01, F=16.30, P <0.001 and F= 6.30, P <0.05). Each mean represents data from eight hairs, error bars represent SEM.

resulted in inflated SBFI ratios. While K^+ does not interfere with the sensitivity of the dye (Minta and Tsien, 1989), it does affect the ratio values if there are large changes of K^+ in the medium (Minta and Tsien, 1989).

Intracellular K^+ content

The root hairs had 55–60 mM K in the cytosol, and this was not affected significantly by external Ca^{2+} (Table 1). This is close to published values for cytosolic K^+ activity obtained by microelectrode measurements, such as 70 mM in barley root cells (Walker *et al*., 1996), 68 mM in leaf epidermal cells of barley, which was reduced to 15 mM by the addition of 200 mM NaCl (Cuin *et al*., 2003), and 65 mM in root protoplasts (obtained by use of PBFI, Lindberg, 1995). It should be noted that subcellular compartmentation of K^+ can be altered by the supply of K^+ (Memon *et al*., 1985); imposition of K^+ deficiency resulted in a concentration of 40 mM in barley root cells (Walker *et al*., 1996). Due to the difficulties of measuring K^+ with PBFI in the presence of Na^+, it was not possible to measure the effects of growing seedlings for several days with NaCl on cytosolic K^+.

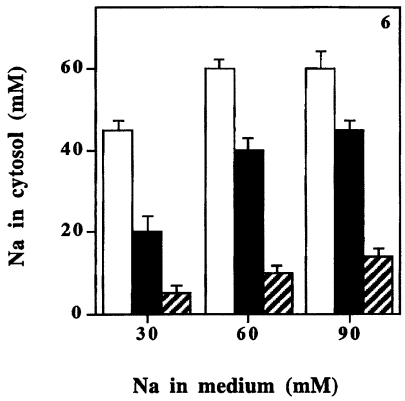

Fig. 6. Accumulation of Na^+ in the cytosol of root hairs of *A. thaliana* grown for 2 d in 30, 60 or 90 mM NaCl and in 5.0 mM Ca^{2+} (stripes), 2.0 mM Ca^{2+} (black), or 0.5 mM Ca^{2+} (white). Sodium, Ca^{2+}, and the interaction had significant effects (F=831.69, P <0.001, F= 218.90, P <0.001 and F= 3.01, P <0.05). Each mean represents data from eight hairs, error bars represent SEM.

Effect of NaCl on Na^+, K^+, and Ca^{2+} accumulation in entire seedlings

To verify that there was a correlation between the results obtained with fluorescent dyes and tissue ion content using a known method (ion chromatography), large amounts of seedlings were grown as in previous experiments, and exposed to NaCl for 2 d. When the seedlings were harvested, the shoots were removed first because they were erect and not in contact with the medium, and it was important to prevent possible contamination from ions in the medium. After the shoots were removed, the roots were removed from the medium. Both organs were rinsed in distilled water briefly to remove external ions loosely bound to the outer parts of the apoplast. The shoot dry mass:root dry mass was 10:1, and the dry mass and total ion mass in the root and shoot fractions were combined, and ion concentration was calculated from the combined values.The fractions were combined because this was an experiment to measure the concentration of Na^+, K^+, and Ca^{2+} in seedlings after 2 d of NaCl stress. The calculations were performed to present ion mass:dry weight mass of the seedling as a measure of the concentration present in the entire seedling, because NaCl stress affects uptake of water by the seedling independently of Na^+ and K^+ uptake, and in a manner which may not be alleviated by additional external Ca^{2+}. Thus, dry mass was used as a normalizing factor because it is more robust to variation from differential uptake of water than is fresh weight. Consistent with the results described above, the more NaCl present in the medium, the more Na^+ was in the seedling, and the 5.0 mM Ca^{2+} treatment contained the least amount of Na^+ at all concentrations, and NaCl had no effect on the amount of Ca^{2+} in the seedlings (Table 2). The presence of Na^+ in the 0 mM treatment is due to Fe-EDTA, which is a ferric-sodic salt, and which contributes 2 μM to the medium. There was little difference between the 0.5

and 2.0 mM Ca^{2+} treatments with respect to Na^+ concentration, although at higher NaCl levels the seedlings grown in 2.0 mM Ca^{2+} contained slightly less Na^+ and at all concentrations of NaCl, K^+ was reduced regardless of external Ca^{2+} concentrations, and the seedlings grown in 5.0 mM Ca^{2+} contained slightly less K^+.

Discussion

These data demonstrate by direct measurement that supplemental Ca^{2+} reduces Na^+ accumulation in the cytosol of salinized root hairs, particularly at the apex. When root hairs which had been loaded with SBFI were exposed to NaCl, an increase of Na^+ activity in the cytosol occurred rapidly during the first 5 min (Fig. 5). After this time, Na^+ activity saturated at a level inversely proportional to the extracellular concentration of Ca^{2+}. The timing of the increase was the same in root hairs grown in 0.5, 2.0 or 5.0 mM Ca^{2+}. This effect lasted for several days (Fig. 6). In the control groups for the experimental treatments, the cytosolic K^+ concentration remained unaltered by external Ca^{2+}. While root hairs are specialized plant cells, and conclusions drawn from studies with root hairs may not be necessarily extrapolated to the response of other plant cell types, root hairs are responsible for greatly increasing root surface area, which makes them very important for water and ion uptake, such as for phosphorus (Bates and Lynch, 2000), and the physiological response of root hairs to NaCl stress can have important implications for the plant as a whole.

These results are consistent with those reported by Davenport and Tester (2000), who described Na^+ transport through a weakly voltage-dependent, non-selective cation channel, present in plasma membranes of wheat (*Triticum aestivum*). They report that Ca^{2+} and Mg^{2+} inhibited Na^+ transport. Demidchik and Tester (2002) have also described non-selective cation channels (NSCCs) in protoplasts from *A. thaliana* roots, and they suggested that NSCCs are a distinct group of plant ion channels, which mediate toxic Na^+ influx to the cell. NSCCs are blocked by Ca^{2+} and other divalent and trivalent cations. Another possible explanation for this study's results is that the increased Ca^{2+} stimulates an efflux of Na^+ from the cytosol. Elphick *et al*. (2001) reported that plants with a mutant *sos*3 locus, which codes for a Ca-sensing protein in the wild type (Quintero *et al*., 2002), had less efflux of Na^+. The inhibitory effects of Ca^{2+} on Na^+ influx through channels in NaCl-stressed plants has been reviewed in Tyerman and Skerrett (1999). In whole-plant studies, Cramer *et al*. (1987) reported that supplemental Ca^{2+} aids in Na^+ exclusion.

Two important points of biological relevance are raised by the data in Fig. 5. One is that root hairs with more Ca^{2+} take up Na^+ with similar kinetics as those grown in low Ca^{2+}, and the final concentration of Na^+ in the cytosol was lower in root hairs grown with higher Ca^{2+}. This may be caused by Ca^{2+} blocking NSCCs, stimulating Na^+/H^+ antiport activity (which is indirectly regulated by Ca^{2+}, Qiu *et al*., 2002) or that additional Ca^{2+} enhances K^+/Na^+ selectivity (Cramer *et al*., 1987). Another is the amount of Na^+ in the cytosol. Root hairs grown in low Ca^{2+} have almost as much Na^+ as K^+ after a few minutes. These quantities could aid researchers who are studying intracellular Na^+ and K^+ in understanding the intracellular environment of salinized plants.

SBFI is useful for measurement of Na^+ in plant cells, because it allows rapid measurements of a dynamic $[Na^+]$ in the cytosol. As far as is known, this is the first report of the use of SBFI to measure Na^+ within the cytosol of plant

Table 1. *Cytosolic K in the root hairs of* A. thaliana *plants grown in increasing concentrations of Ca*

There was no significant difference between the treatments, each mean represents measurements from at least five hairs over three different plants, numbers in parenthesis represent SEM.

Ca in medium (mM)	K in cytosol (mM)
0.5	54 (5)
1.0	50 (5)
2.0	50 (4)
4.0	58 (3)
5.0	56 (4)

Table 2. *Sodium (Na^+) and potassium (K^+) content (mg g^{-1} dry weight) of* A. thaliana *seedlings that have been exposed to increasing concentrations of NaCl for 2 d*

Sodium and calcium had significant effects on the accumulation of sodium (F=76.477, P <0.001 and F=6.388, P <0.01, respectively) and potassium (F=12.283, P <0.001 and F=5.093, P <0.01), and there was no significant interaction between these variables. Only calcium had a significant effect on calcium accumulation (F=56.575, P <0.001), and only the control values are shown. Numbers in parenthesis represent SEM and n=4, one sample=combined root and shoot dry masses from a tissue culture dish containing 200 seedlings.

Ca^{2+} in medium (mM)	0 mM NaCl			30 mM NaCl		60 mM NaCl		90 mM NaCl	
	Ca^{2+}	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
0.5	2.6 (0.2)	8.7 (1.1)	57.0 (4.6)	26.1 (2.6)	48.9 (2.8)	37.2 (3.7)	47.7 (4.6)	47.1 (4.2)	35.7 (3.6)
2.0	4.3 (0.3)	8.7 (1.6)	60.0 (2.5)	26.6 (2.1)	44.4 (0.8)	33.1 (2.3)	41.9 (6.1)	43.3 (5.9)	39.3 (5.1)
5.0	7.6 (0.4)	4.7 (0.5)	49.2 (2.5)	18.2 (1.2)	34.8 (0.6)	27.4 (2.0)	39.8 (2.3)	38.7 (5.4)	28.7 (4.1)

cells, and this application complements the recently reported use of SBFI to measure apoplastic Na^+ (Mühling and Läuchli, 2002). This method not only allows rapid measurements, but allows for greater spatial resolution than intracellular microelectrodes. The loading process did not inhibit root hair growth, and there is negligible autofluorescence from the extended portion of the root hair. There were no rapid changes in dye fluorescence intensity in the cytosol for at least 4 h, which constitutes a large part of the stationary growth phase of an elongating root hair (Halperin *et al*., 2003). The drawbacks are that it is dim, and that large changes in K^+ can affect Na^+ measurements. Also, SBFI and PBFI are not suitable for measurements of vacuolar Na^+ and K^+ because of the acidic pH (Mühling and Läuchli, 2002; A Minta, personal communication). When the loading solution of the dye was pH 7, very little dye entered the root hair, and adequate loading occurred only at pH 8. Dye movement from the cytosol to other compartments does not necessarily affect the validity of measurements, as reported by Brauer *et al*. (1995). This is because emitted signals from two different excitation wavelengths are ratioed, thus variability resulting from such factors as dye concentration, illumination intensity, emission collection efficiency, and effective cell thickness in the optical beam are cancelled out by division of the two signals (Grynkiewicz *et al*., 1985). An important property of root hairs which can affect certainty of dye localization is the small fraction of cell volume occupied by the cytoplasm. The cytoplasm and most of the organelles are concentrated at the tip (Peterson and Farquhar, 1996). When a healthy root hair is observed under the microscope, the cytosol is evident as a streaming substance flowing along the sides of the shaft. Because of the constant motion, the cytosol and, consequently, any dye signal associated with the cytosol yields a different image from second to second. This means that the distribution of cytosol of a root hair imaged under bright field will have changed by the time a fluorescent image can be taken, and the cytosol is streaming as the images are being taken. Thus, single-wavelength images of fluorescence from root hairs which contain dye will show a slightly different dye distribution than cytosol distribution in the bright-field image. This effect is seen in Figs 1a and b and 2a and b, in which the dye signal appears to be spotty and not completely consistent with the presence of the cytosol, which is faintly demarcated from the vacuole by faint black lines resulting from slightly different optical properties. This can be the cause of the patchy appearance in the SBFI ratio image (Fig. 1c), or it may be that Na^+ is not uniformly distributed in the cytosol. PBFI is useful in experiments in which the plant will not encounter a significant amount of Na^+. As a ratiometric dye, it undergoes a very slight ratiometric shift when compared to other dyes (Minta and Tsien, 1989). Also, in practice, imaging PBFI was difficult because it is a dimmer dye than is SBFI, and did not load as efficiently. Because of the weak signal (from PBFI), more variation can enter into experiments.

Other studies for which these methods would be useful are to measure the uptake of other salts of Na^+, and to monitor Na^+ or K^+ uptake in mutants such as *sos*3. As these mutants are used in K^+ uptake studies and NaCl studies, these dyes offer researchers means to measure Na^+ uptake and K^+ dynamics in living cells, in real time. There is also a potential for the use of these dyes with various cation transporters. SBFI and PBFI measurements were compared with measurement of Na^+ and K^+ in whole *A. thaliana* plants exposed to similar conditions. In common with the data from SBFI, 5.0 mM Ca^{2+} was most effective in assisting Na^+ exclusion, though unexpectedly there was little difference between 0.5 and 2.0 mM Ca^{2+}. This discrepancy may exist because SBFI measurements of Na^+ in the cytosol do not include vacuolar Na^+ concentrations, apoplastic Na^+ concentration, and Na^+ uptake along the entire length of the root. A whole plant consists of many cell types and has different uptake dynamics than a root hair, there are differences in osmotic potential from the root to the shoot, and the apoplast of plants are negatively charged, due to the cell walls, which can bind Na^+. Surprisingly, supplemental Ca^{2+} did not prevent loss of K^+, nor did NaCl affect Ca^{2+} concentration, but this may be because this experiment was of a very short duration compared to other experiments with whole plants (Guerrier, 1996; Cramer *et al*., 1987). Guerrier (1996), who used a 4 d time-point as the shortest treatment, reported that after 4 d the salt-tolerant *Lycopersicon pimpinellifolium* increased uptake of K^+ and Na^+, whereas the salt-sensitive *L. esculentum* had reduced K^+ after the first day. After 15 d, both species exhibited similar losses of K^+.

Because fluorescent measurements with SBFI were consistent with differences that would be expected between plants grown at 0.5 mM and 5.0 mM, as well as with results that have long been known about the beneficial effects of Ca^{2+} on keeping Na^+ out of plant cells, the authors have confidence in the validity of the measurements. These dyes can be powerful tools in salinity research because it is now possible to measure cytosolic Na^+ dynamics in living cells, and image Na^+ distribution within the cytosol, which can be used to determine some of the environmental factors which can help alleviate the ionic problems associated with salt stress.

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