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Osmotic Sensitivity of Ca^{2+} and H^+ Transporters in Corn Roots: Effect on Fluxes and Their Oscillations in the Elongation Region

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Abstract. Seedling roots of corn were treated with different concentrations of mannitol-containing solution for 1 to 1.5 hr, and net fluxes of Ca^{2+} and H^+ were measured in the elongation region. H^+ fluxes were much more sensitive to osmotic pressure than were Ca^{2+} fluxes. Oscillations of 7-min period in H^+ flux, normally observed in the control, were almost fully suppressed at high osmotic concentrations. Net H^+ flux was shifted from average efflux of 25 ± 3 nmol m^{-2} sec^{-1} to average influx of 10 ± 5 nmol m^{-2} sec^{-1} after the incubation in 100 mM mannitol. The larger the osmotic concentration, the larger was the H^+ influx. This flux caused the unbuffered solution of pH 4.85 to change to pH 5.3 after mannitol application. It appears that the osmoticum suppresses oscillatory H^+ extrusion at the plasma membrane. Discrete Fourier Transforms of the H^+ flux data showed that, apart from suppression of the 7-min oscillations in H^+ flux, mannitol also promoted the appearance of faster 2-min oscillations. Ca^{2+} influx slightly increased after mannitol treatment. In addition the 7-min oscillatory component of Ca^{2+} flux remained apparent thereby showing independence of H^+ flux.

Key words: *Zea mays* — Ion transporters — Osmotic stress — Hydrogen — Calcium — Mechanosensory channels — Oscillations

Introduction

Osmotic pressure is one factor affecting properties of membrane ion transporters. High osmotic concentrations in the external solution reduce water availability to roots. As the turgor pressure is a major driving force for cell growth by expansion, plant cells change to restore the original turgor after being subjected to osmotic stress (Frensch & Hsiao, 1995; Lew, 1996). This osmotic adjustment may be achieved by various combinations of changes in ion pump rates and in passive permeability (Levitt, 1972; Bisson & Gutknecht, 1977).

It seems that ionic mechanisms of osmotic adjustment vary significantly between different species. Decreasing turgor by raising the external osmolarity stimulated the active uptake of K^+ in *Valonia* and Cl^- in *Halicystis* (Gutknecht, Hastings & Bisson, 1978). Alterations in cytoplasmic Ca^{2+} concentrations were suggested as a part of the turgor signal transduction chain for *Lamprothamnium* (Okazaki & Tazawa, 1990) and *Chara* (Bisson et al., 1995). A ubiquitous component of osmotic adjustment in higher plants is modulation of the proton-pumping activity (Rubinstein, 1982; Reinhold, Seiden & Volokita, 1984; Li & Delrot, 1987). Passive H^+ transport is also affected (Bisson & Gutknecht, 1977; Rubinstein, 1982; Lew, 1996) implying a multicomponent osmo-regulatory system (Berrier et al., 1996).

Although it is difficult to distinguish between turgor-sensing and osmo-sensing (Lew, 1996), one possible mechanism of osmotic adjustment may involve stretch-activated channels (SAC) in the plasma membrane (Ramahaleo, Alexandre & Lassales, 1996; Lew, 1996; Cui & Adler, 1996; Berrier et al., 1996). However, all reported evidence of SAC was obtained by using the patch-clamp technique. This imposes artificial conditions on the patched membrane, and raises questions about possible artefacts (Morris & Horn, 1991; Ramahaleo et al., 1996). There is a need for more experimental observations of SAC effects at the tissue or organ level, measured by a noninvasive technique. Long-term microelectrode measurements on a single cell of a fast growing root is practically very difficult. For this reason we regard our noninvasive MIFE system to measure ion fluxes as an ideal tool for research on ion transporters.

We have already described oscillations in net H^+ and

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Ca^{2+} fluxes around the elongation region of corn roots (Shabala, Newman & Morris, 1997). The fast and slow components of these oscillations reflected the biophysical and biochemical mechanisms of plant pH homeostasis, and were supposed to belong to active and passive H^+ transporters, respectively. The oscillations correlated with root nutation and may be involved in the mechanism of redistribution of growth substances around the root circumference in the elongation region (Shabala & Newman, 1997). Both oscillations also showed a tight link with root growth. When elongation ceased, so did oscillations and root nutation. These observations illustrate the physiological significance of ion flux oscillations for root metabolism. As osmotic stress affects the growth process, we might expect that parameters of these oscillations will be affected by applied osmotica. Do all ion transporters have similar sensitivity to applied osmotic pressure, or do hyperosmotic conditions selectively modify some of them?

In this study we measured fluxes of H^+ and Ca^{2+} as two major ions involved in the process of cell elongation. Our observations show that H^+ ion transporters are more affected by applied osmotic pressure than Ca^{2+} ones. This effect appeared mainly in the suppression of active H^+ pumping and in shifting the measured H^+ flux to net influx. Apart from suppression of the 7-min oscillatory component of H^+ flux, normally observed in control (Shabala et al., 1997), mannitol also promoted the appearance of faster oscillations of 2-min period. We suggest that they are due to the presence of mechanosensory channels being activated by changed turgor.

Materials and Methods

Plant Material and Preparation

Corn seeds (*Zea mays* L. cv Aussie Gold) were grown and prepared for measurements essentially as described by Shabala et al. (1997). On the third day, when the root length reached 60 to 80 mm, plants were decapped and transferred into the measuring chambers 1 to 1.5 hr before the flux measurements. In the control variant, the bath solution was unbuffered 0.2 mM $CaSO_4$.

When transient responses to mannitol were studied, 1 mL of 1 M mannitol made up in 0.2 mM $CaSO_4$ was added to the measuring chamber which contained 9 mL of the bath $CaSO_4$. The addition was made by a Pasteur pipette 10 mm from the measured region. Then the bath solution was thorough mixed by sucking and expelling it from the pipette about 20 times. The time required for mannitol addition, mixing, and establishing the diffusion gradients was about 2 min and was discarded from the analysis. For a few measurements, to test another osmoticum, sorbitol was used instead of mannitol.

In experiments with osmotic pretreatment, roots were preincubated in the different mannitol concentrations (from 100 to 300 mM), made up in the basic 0.2 mM $CaSO_4$ solution, from the time when the roots were mounted in the measuring chamber 60–80 min before starting measurements.

Flux Measurements

Net fluxes of H^+ and Ca^{2+} ions were measured noninvasively using the Tasmanian MIFE™ system (Unitas Consulting, Hobart, Australia) as described by Shabala et al. (1997). Additional information about specification, characteristics and commercial availability of the MIFE system are also provided on the internet at ''http:/www.phys.utas.edu.au/physics/biophys/mife.htm.'' Briefly, the electrode tips were filled with specific ion-selective resins (catalogue number 82500 for H^+ and 21048 for Ca^{2+}, Fluka Chemical, Buchs, Switzerland), calibrated, and mounted on the multi-micromanipulator. One H^+ electrode and one Ca^{2+} electrode were used at the same time. The electrode tips were positioned, with 3–5 μm spacing, in a line parallel to the root axis, 50 μm above the root surface. During measurements, the distance between the root surface and the electrodes was changed from 50 to 90 μm with a frequency 0.1 Hz. The difference in electrochemical potential between these two positions was measured, and the flux of each ion was derived from these measurements assuming cylindrical geometry diffusion (Newman et al., 1987; Shabala et al., 1997).

The total time of measurements was 2 hr for the control and for 100 mM mannitol; one hour for all other variants. All measurements were done for the elongation region, 5 mm from the root tip. As plants showed a high rate of growth and strong nutational movement in this region (Shabala & Newman, 1997), constant correction of the electrode position was essential. We followed the same cell during the root growth using the three-axis hydraulic manipulator as described by Shabala and Newman (1997).

Spectral Analysis of Data and Statistics

Various methods of data analysis in the time domain have been described for analysis of oscillations in biological systems (Robinson, Rust & Scott, 1979; Zachariassen et al., 1987; Antonsen et al., 1995; Shabala & Newman, 1997). Every biological experiment contains a limited number of data points usually obtained at discrete time intervals. As most analytical methods are designed for continuously varying data over an infinite time interval, each method has its advantages and limitations for application to biological systems.

In this research we have chosen the standard Fourier analysis package, available in EXCEL 4.0, and based on the Fast Fourier Transform (FFT), as a convenient mathematical tool. For our finite 'data window', the Discrete Fourier Transform (DFT) is used instead of the continuous transform. Briefly, if there are n data points at intervals τ during a total time $T = n\tau$, the data series $f(t)$ is replaced by a trigonometrical sum of harmonics

$$S(t) = \frac{a_o}{2} + \sum_{1}^{n} f(t)\cos 2\pi\nu t + \sum_{1}^{n} f(t)\sin 2\pi\nu t$$

Using the IMABS and IMARGUMENT tools in EXCEL 4.0, we obtained the moduli and phases of the complex amplitudes returned by DFT application. These moduli were later plotted against the period of the harmonic components for the discrete frequencies $\nu = 0, 1/T, \ldots, (n-1)/T$.

Because our measurements represented a data window, we used a number of steps of data processing before the final spectral analysis (*see* Fig. 1). For each variant (control, 100 mM and 200 mM mannitol), we had from 6 to 8 individual plants measured and selected for spectral analysis. Each of them provided records of the net ion fluxes (Ca^{2+} and H^+) and of ion concentrations every 10 sec. The sample size was about 720 for the two hours of measurements. Because the DFT requires a sample size which is a power of 2, these 120-min data intervals were divided into 5 overlapping time intervals of about 42.6 min, each con-

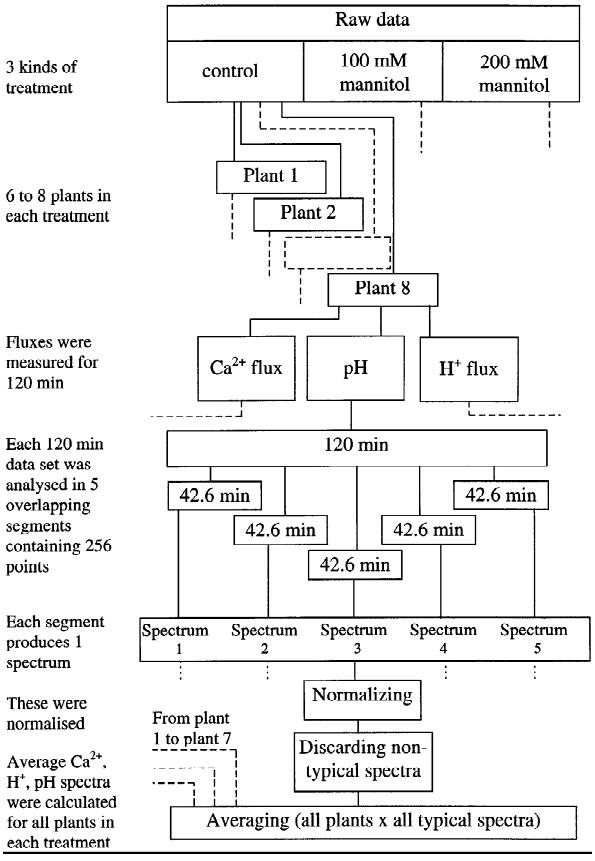

Fig. 1. Diagramatic illustration of the data preparation and spectral analysis by the Discrete Fourier Transform as described in Materials and Methods.

taining 256 data points. This is shown diagrammatically in Fig. 1. The reason for using the overlapping intervals is that the DFT is unreliable at the ends of the data window. Valuable information could thereby be lost for some time intervals. Our overlapping data windows put the data from the end of the previous data interval into the middle of the next interval and thus made it possible to minimise error in the results obtained. For the sake of reliability, we also ignored the two components of the lowest frequency in the resulting Fourier spectrum. As the number of data points was 256 for each spectrum, the first reliable component appeared at 14.2 min. As we expected to find the maxima in the Fourier spectra for periods around 7 min (*see* Shabala et al., 1997), the rejecting procedure was acceptable, and we do not consider any oscillations that may have been present with periods greater than 20 min.

The next stage was to filter the data. After spectra for the five overlapping time intervals of about 42 min were obtained for each root, all these spectra were normalized. Then all spectra showing no oscillations were discarded. The discarding procedure, of less than 15% of the spectra, was a necessary step in the data analysis, because fast oscillations do not appear in every time interval for the plants selected for analysis. The Fourier spectrum of a time interval without visible oscillations represents 'white noise', which reduces accuracy of the analysis when averaged together with other spectra. Most of these discarded spectra corresponded to the specific time interval when the slow H^+ flux component approached its maximum and the fast 7-min oscillations ceased. A typical example suitable for such discarding

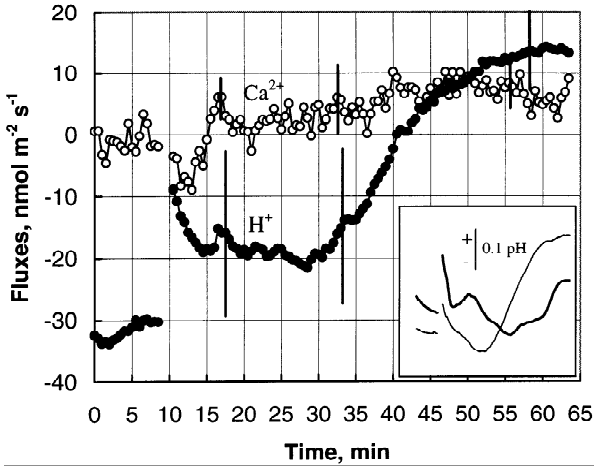

Fig. 2. Transient responses of H^+ (closed symbols) and Ca^{2+} (open symbols) net fluxes to hyperosmotic treatment (100 mM mannitol applied at 8 min). Mean fluxes ($n = 4$ for H^+ and $n = 6$ for Ca^{2+}) are shown. Measurements were made at 5-sec intervals. Each point represents the average value of the six measurements made during 30 sec. The insert illustrates the typical course of pH changes for two individual plants after mannitol application. Error bars are SE.

may be seen in the range 40–55 min in Fig. 3*A* here or, more clearly, in time interval from 50 to 100 min shown by Shabala et al. (1997, Fig. 3*A*). There was no apparent correlation between discarded spectra and any specific treatment applied. Twenty to 30 spectra from a total of 6 to 8 individual plants were included in the averages after the discarding process.

Results

Transient Responses to Osmotic Treatment

The average value of the net H^+ flux in the elongation zone of control plants is significantly negative (Shabala et al., 1997). This strong efflux caused the pH averaged over 50 to 90 μm from the root around the measured site to be slightly more acid than the bulk solution (pH 5.2). Average net Ca^{2+} flux in control is close to zero in the elongation zone.

Addition of 100 mM mannitol into solution caused immediate transient responses in both H^+ and Ca^{2+} transport (Fig. 2). Different plants measured might be at different stages of the slow oscillatory curve (Shabala et al., 1997), so variability of the flux patterns was very large. Nevertheless, the qualitative character of these transient responses was similar for all plants (Fig. 2).

Three distinct phases can be seen in the ion flux transient responses to mannitol addition. The first phase started immediately after mannitol application and lasted a few minutes. There was discontinuous pH rise of about 0.1–0.2 pH units immediately after mixing was over (Fig. 2 insert). H^+ efflux decreased immediately, and

Table 1. H^+ and Ca^{2+} fluxes (nmol m^{-2} sec^{-1}, net influx positive) and local pH around the elongation zone of corn roots as a function of mannitol concentration

Concentration, nM	H^+ flux	Ca^{2+} flux	pH
Control	−24.9 ± 3.6 (16)	4.6 ± 1.3 (16)	4.81 ± 0.02 (16)
100	10.2 ± 5.0 (12)	8.41 ± 1.1 (12)	5.19 ± 0.02 (12)
200	28.4 ± 9.0 (6)	32.7 ± 4.9 (6)	5.23 ± 0.01 (6)
300	29.8 ± 6.4 (6)	10.3 ± 1.4 (6)	5.34 ± 0.04 (6)

Means ± SE (sample size) are shown.

Ca^{2+} showed significant efflux for a short time (Fig. 2). Average Ca^{2+} concentration was not strongly affected (*data not shown*).

During the next phase, of 15- to 40-min duration, all parameters measured showed complex multiphase kinetics. For each root, H^+ efflux tended to increase for a while, showing a series of fast oscillations. Ca^{2+} concentration in the bath near the root surface started to decrease, consistent with the developing Ca^{2+} influx. Because the results shown in Fig. 2 represent the average of 7 individual plants, many specific features of these transient responses are masked.

In the third phase of the transient curve, there were slow monotonic rises in both H^+ and Ca^{2+} fluxes (Fig. 2). The average net H^+ flux became an influx approximately 40 min after mannitol application and caused the pH to increase up to 0.2–0.3 units higher than the control. There was a small but rather steady Ca^{2+} influx (Fig. 2) at the end of the transient response. Ca^{2+} concentration decreased by 10–40 μM in one hour. The transient response caused by mannitol application lasted 60–80 min.

Similar results were obtained using 100 mM sorbitol (*data not shown*), indicating the flux changes were caused by alteration in the external osmotic pressure and not by the specific properties of mannitol.

Steady Fluxes After Pretreatment in Mannitol

As is evident from Fig. 1, 1–1.5 hr was enough for the plant to complete adjustment to the osmoticum. As a result of this adjustment, both H^+ and Ca^{2+} fluxes were significantly different from control. There was a clear shift of the average net H^+ flux from efflux to influx (Table 1), and the Ca^{2+} flux tended to become more positive. For H^+ this shift was apparently dependent on mannitol concentration. The link between osmotic concentration and increase in the Ca^{2+} influx was not so clear. The change of net H^+ flux from efflux to influx at high osmotic concentrations, caused pH around the measured region to increase to values even higher than the bath solution pH (Table 1).

Slow Oscillatory Component of Ion Fluxes

Normally, two distinct oscillatory components of H^+ flux can be observed in the elongation region of corn roots (Shabala et al., 1997). An example of these oscillations for a representative control plant is given in Fig. 3*A*. One of these components is a fast oscillation with period of about 7 min. This fast oscillation is modulated by a slow, rhythmic change with period of about 1.5 hr. This modulation caused H^+ flux to be changed from net efflux to influx, and back again. For Ca^{2+} flux, the fast oscillation is similar to that of H^+ but slightly advanced in phase. The slow Ca^{2+} oscillatory component normally appears as a modulation of the amplitude of the fast Ca^{2+} flux oscillation around some steady level of Ca^{2+} flux (Fig. 3*A*).

Osmotic pretreatment caused regular changes in both oscillatory components of the ion fluxes. Fig. 3*B* shows one representative example of flux behaviour for a plant treated with 100 mM mannitol. The statistics are given in Table 2. The slow oscillatory components of H^+ and Ca^{2+} fluxes remained present after osmotic pretreatment (Fig. 3*B*). Their temporal characteristics were similar to those in the control, but the amplitude characteristics were not (Table 2). The amplitude of the slow H^+ flux oscillations was 0.4 of the control amplitude.

Fast Ion Flux Oscillations

The most significant changes caused by osmotica seem to appear in the fast oscillatory component of ion fluxes. Fast oscillations in H^+ and Ca^{2+} flux showed very different sensitivity to the applied osmoticum. Mannitol almost completely suppressed the 7 min H^+ flux oscillations, whereas the fast Ca^{2+} component could be clearly seen (Fig. 3*B*). Where there was some evidence of 7 min H^+ flux oscillations, their amplitudes were about 10 times smaller than in the control; for Ca^{2+} oscillations this factor was less than 2 (*data not shown*). For every plant, after mannitol treatment the fast oscillations in H^+ flux were more noisy than in the control and possessed a very small amplitude, normally much less than for calcium, whereas in the control the amplitude ratio of H^+/Ca^{2+} fluxes is normally about 1.2 (Shabala et al., 1997).

Another remarkable regularity of the osmotic effect on ion transporters was the presence in the H^+ flux of oscillations of much shorter period than 7 min (Fig. 3*B*). These oscillations were also very irregular, containing more than one fast oscillatory component. Figure 4 shows these oscillations more clearly by means of the pH changes of two individual plants under control conditions (*A*) and in 100 mM mannitol (*B*). There is no evidence of any oscillatory component other than 7 min in Fig. 4*A*. However, we can clearly see a wide range of oscillations after osmotic treatment (Fig. 4*B*). These in-

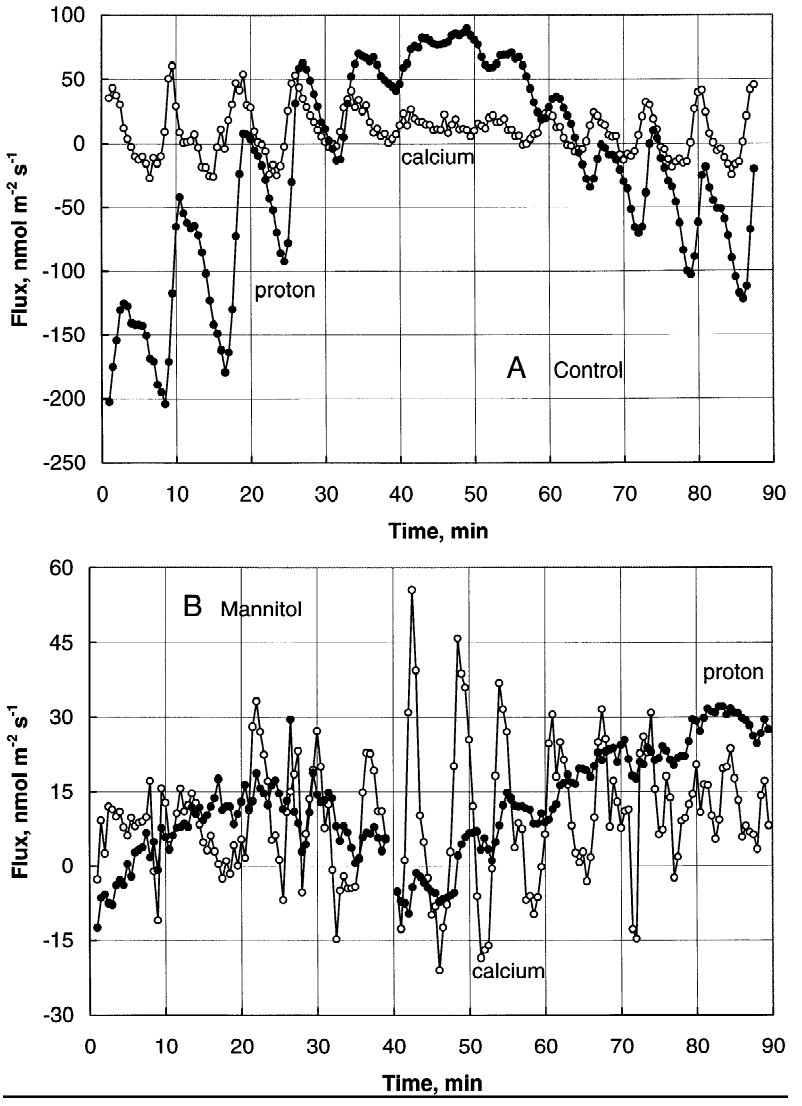

Fig. 3. H^+ (closed symbols) and Ca^{2+} (open symbols) net fluxes around the elongation region of corn roots. Representative records from one individual plant for control (*A*) and 100 mM mannitol (*B*) variants are shown. Measurements were made at 5-sec intervals. Each point represents the average value of the six measurements during 30 sec. Mannitol treatment strongly suppressed the fast oscillatory component of the H^+ flux.

Table 2. Osmotic effect on parameters of slow H^+ flux oscillations

Parameters	Control	Mannitol
Half-period of slow H^+ oscillations, min	44.5 ± 5.2 (n = 7)	50.2 ± 5.4 (n = 12)
Half-period of slow pH oscillations, min	45.33 ± 5.3 (n = 12)	45.4 ± 5.4 (n = 12)
Average amplitude of slow H^+ oscillations, nmol m^{-2} sec^{-1}	131.2 ± 20 (n = 16)	55.8 ± 6.4 (n = 12)*
Average amplitude of slow pH oscillations, pH units	0.12 ± 0.02 (n = 6)	0.10 ± 0.02 (n = 12)

Plants were pretreated with 100 mM mannitol 1 hr before measurements. Mean ± SE (sample size), *$P < 0.01$, by Students' t Test.

clude those close to 5–7 min (plant 2 marked with the open circles) as well as faster 1.5–2 min oscillations (plant 1, dark diamonds in Fig. 4*B*).

As visual observations are not a precise tool in making these comparisons, we applied the spectral analysis procedure to find the quantitative parameters of the fast H^+ and Ca^{2+} flux oscillations caused by osmotic treatment. The DFT was applied to the data series and results are shown in Fig. 5. In general, both H^+ flux and pH spectra showed qualitative identity. The pH changes represent a physical averaging of data from a large group of neighboring cells and over time, which minimizes noise and the influence of external factors. For this reason, we show here the average Fourier spectra of the pH changes (Fig. 5*A*) to characterise the proton transporters in the plasma membrane. The number of oscillatory

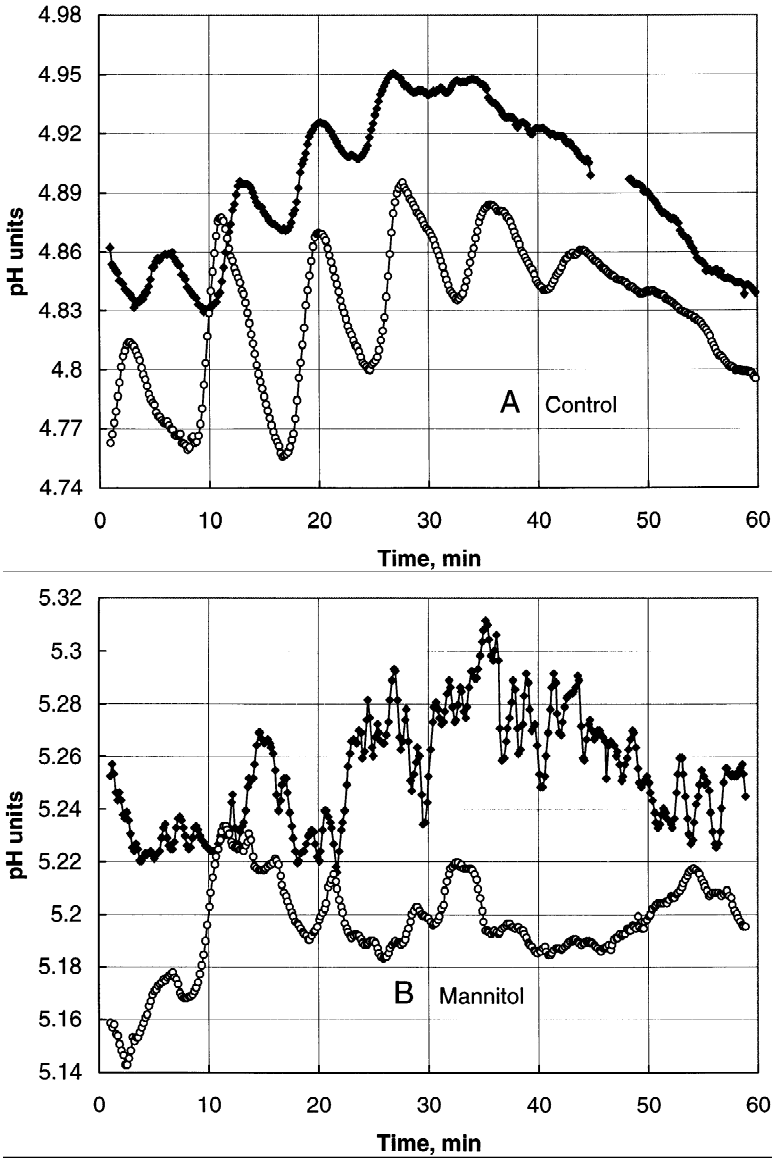

Fig. 4. Oscillations in local pH caused by H^+ fluxes around the elongation region for control (*A*) and 100 mM mannitol (*B*) variants. Two representative plants for each variant are shown. The 7-min oscillatory component is apparent for control (*A*); a wide range of oscillations was observed after osmotic treatment (*B*). Each point is average pH over 10 sec.

components available in the Fourier spectra was 128 (the Nyquist frequency corresponds to a component with period about 0.33 min).

Results of the spectral analysis (Fig. 5*A*) are consistent with our visual observations (Figs. 3, 4) of mannitol suppression of the fast 7 min component of H^+ flux oscillations. There is a distinct sharp peak around 7 min in the Fourier spectrum in the control. After mannitol treatment this peak is no longer distinguishable. Instead, a statistically significant spike of period around 2 min appears. At the same time, the 7-min peak for Ca^{2+} flux oscillations, shown in the control is still traceable after osmotic treatment (Fig. 5*B*). No other significant component can be observed in the Ca^{2+} Fourier spectra, although there is some evidence of a shoulder from 0.5 to 3 min for Ca^{2+} flux as well.

Discussion

Results reported here seem to be the first long-term measurements of the Ca^{2+} and H^+ flux kinetics induced by hyperosmotic treatment. The temporal resolution of the MIFE system makes it possible to study the very early stages of root responses to increased osmotica as well as revealing the fast oscillations in ion flux behaviour induced by hyperosmotic treatment.

Changes of turgor pressure could alter the thickness of the plasma membrane, resulting in altered membrane activity (Coster, Steudle & Zimmermann, 1976). Changed turgor causes changes in membrane potential (Racusen, Kinnersley & Galston, 1977; Zimmerman & Becker, 1978; Pantoja & Willmer, 1986) and in membrane resistance and permeability (Jenkinson & Scott,

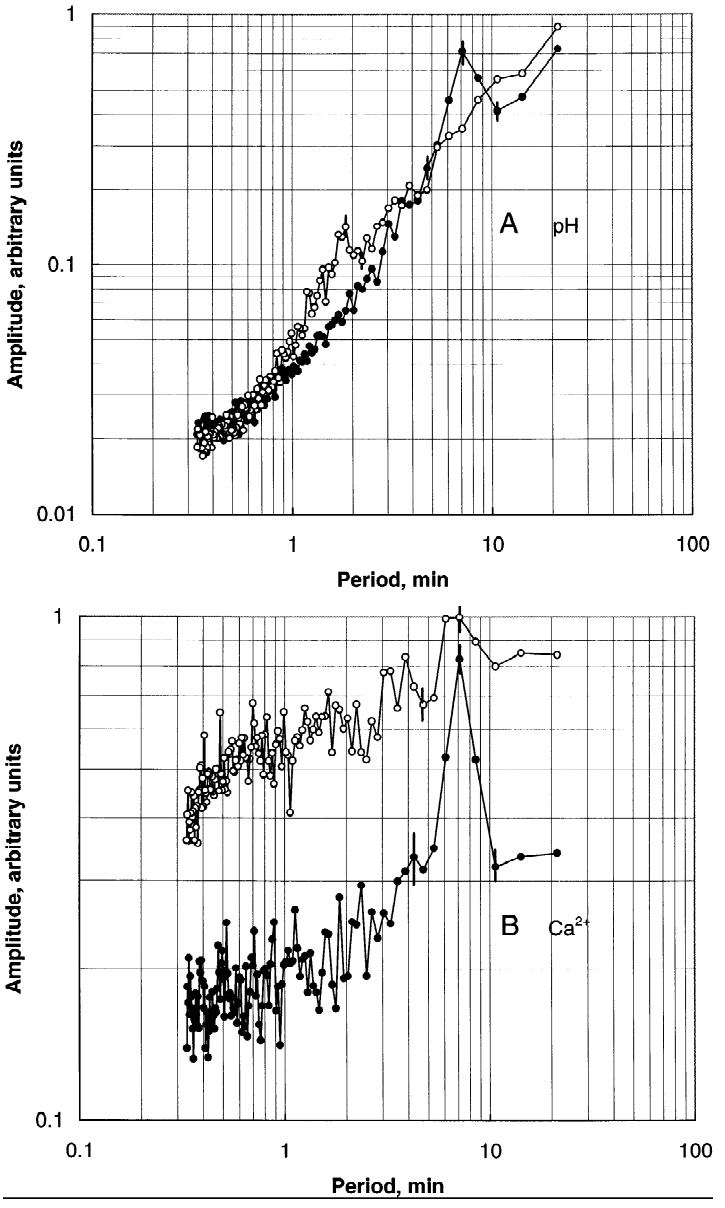

Fig. 5. Discrete Fourier Transform of pH (*A*) and Ca^{2+} flux (*B*) oscillations in control (dark symbols) and after 10 mM mannitol treatment (open symbols). The spectra are the means of the normalised spectra calculated as shown in Fig. 1. A sharp 7-min peak is clearly seen in the control for pH (*A*), but is fully suppressed after mannitol application. Instead, a statistically significant spike appears at just under 2 min. Unlike pH, Ca^{2+} flux spectra still show the 7-min peak after mannitol treatment although the peak is far less sharp, being surrounded by broad-band ''noise'' (*B*). Error bars (SE) are shown for the crucial points.

1961; Pantoja & Wilmer, 1986; Lew, 1996). However, the strongest osmotic effect seems to appear in the modification of active H^+ transport systems (Rubinstein, 1982; Giaquinta, 1983; Reinhold et al., 1984; Li & Delrot, 1987). The complexity of the osmotic effect on both passive and active ion transporters is expected to result in complex ion flux kinetics under hyperosmotic conditions.

We observed several distinct phases in the measured flux transient responses to osmotica (Fig. 2). We interpret them assuming that osmotic inhibition of the active H^+ extrusion consists of two stages. In the first stage there is only partial suppression of H^+ pumping (perhaps a direct turgor effect). In the second stage, osmotic inhibition of the active H^+ transport is mediated by metabolic changes in the cell (an indirect turgor effect).

The *first phase* of the transient response to mannitol (Fig. 2) lasts only a few minutes and is characterized by a sudden pH jump immediately after mannitol application, a sharp decrease of the net H^+ efflux and brief short-term increase in net Ca^{2+} efflux. Because of its rapidity after osmotic change, we suggest that this phase reflects a direct effect of reduced turgor on ion transport in roots.

The observed pH changes may be explained easily as the result of solution mixing. Due to the normal H^+ efflux at our measuring location, the normal pH near the root is below the bulk solution pH. The mixing of the

solution after mannitol addition caused the pH to return to near its value for the bath solution (near 5.2).

There is also an instant turgor effect causing reduction in the net H^+ efflux. Both decreased H^+ pumping or increased passive H^+ uptake may contribute to this reduction. However, as the cytosolic pH of root cells is close to 7.0, the external pH rise caused by the solution mixing would reduce $\Delta\mu_H$ and make passive H^+ uptake smaller, not larger.

The *second phase* is characterized by a transient increase of the net H^+ efflux. As some time may be required for osmotically induced metabolic changes to cause inhibition of the active H^+ transport, the net H^+ efflux may first temporarily increase as a result of the partial reduction of the $\Delta\mu_H$. This phase of increasing H^+ efflux varied between different plants and may last from 15 to 40 min. The magnitude of the increase also varied, sometimes resulting in ''overshoot'' of the both H^+ flux and pH. Behavior of the Ca^{2+} transporters was similar to those for H^+.

The *third phase*, of 30–40-min duration, is the developing suppression of the active H^+ extrusion, so that there was net H^+ influx at the end of the transient response. It may reflect the osmotic inhibition mediated by metabolic changes in the cell. Ca^{2+} influx was also significant (Fig. 2), reducing bath Ca^{2+} by 10–40 μM.

The time course of the observed transient flux changes is in good agreement with reports from other workers. An electrical response of the plasma membrane was reported to begin within 10 min after changed osmotic concentration and to continue for 20 to 30 min (Li & Delrot, 1987). Frensch and Hsiao (1995) showed that a small increase in osmotic pressure (0.1 MPa) temporarily decreased cell turgor and growth, which recovered fully in 5 to 10 min. Under stronger changes (up to 0.6 MPa) elongation stopped for up to 30 min and then resumed at lower rates. In our experiments we used 100 mM mannitol which corresponded to about 0.25 MPa at 25°C, and the transient response was completed by 50–80 min after osmotic application.

From these results it is likely that the main effect of applied osmotica is on the active H^+ extrusion at the plasma membrane. We have previously proposed that the fast 7-min oscillatory component of the H^+ flux oscillations around corn roots relates to electrogenic H^+ extrusion; the slow component relating to changes in passive membrane permeability to H^+ (Shabala et al., 1997). In 100 mM mannitol the 7-min component was almost fully suppressed (Fig. 5*A*). Oscillations were noisy and irregular and possessed very small amplitudes (usually less than 5 nmol m^{-2} sec^{-1}, Fig. 3*B*). At the same time, the average net H^+ flux during two hours was essentially inwards and the slow oscillatory component of the H^+ flux remained clear (Fig. 3*B*) with its temporal characteristics close to those in the control (Table 2). All these indicate that osmotica suppress the H^+-ATPase activity and shift the balance from active extrusion pumping to passive inward leakage of H^+. The results support our supposition that oscillations of 7-min period are attributable to the active H^+ transport systems, and that the slow H^+ flux changes relate to rhythmical changes in passive H^+ transport systems (which are not so strongly affected by mannitol).

Two further observations must be discussed here. The first is the relatively weak effect which mannitol has on Ca^{2+} fluxes. Fast Ca^{2+} flux oscillations could be clearly observed (Fig. 3*B*). Their amplitudes in mannitol were many times larger than those for H^+ flux oscillations, whereas in the control the ratio is close to 1. This indicates that the measured Ca^{2+} flux in mannitol is more than just the result of proton-calcium ion exchange in the cell walls according to the ''weak acid Donnan-Manning'' model (Richter & Dainty, 1989, 1990; Ryan, Newman & Arif, 1992; Arif & Newman, 1993). Hence, our present observations of the osmotic effect on ion fluxes confirm the existence of an intracellular Ca^{2+} oscillator in corn roots (Shabala et al., 1997). At the same time, the average Ca^{2+} influx increased after root treatment with mannitol (Table 1), despite the relatively stable fast oscillations in Ca^{2+} flux.

The second observation of interest is the appearance of an additional peak at just under 2 min in the Fourier spectrum of pH changes in mannitol (Fig. 5*A*). This 2-min component was not present under normal osmotic pressure either in the control or in a range of pH values tested (from 4.0 to 6.0; *data not shown*). This component must be a response of plant tissues to the metabolic changes induced by the mannitol treatment.

We can only speculate about how hyperosmotic treatment affects ion transport systems and modifies their oscillatory behaviour. One possible explanation of the 2-min oscillatory component of ion fluxes may come from the existence of mechanosensory, or stretch-activated, ion channels in the plasma membrane. Several types of mechanosensory Ca^{2+} channels exist in plants (Cosgrove & Hedrich, 1991; Schroeder & Thuleau, 1991; Pickard & Ding, 1993; Bush, 1995). These SAC have been suggested to act as osmoregulatory feedback sensors for mechanical perturbations in plants (Schroeder & Hedrich, 1989; Shroeder & Thuleau, 1991). Tyerman (1992) reported on mechanosensory anion-permeable channels. All of them may interact with H^+ transport systems through cytoplasmic pH (Felle, 1988; Tyerman, 1992; Bush, 1995). Walker et al. (1995) proposed the existence of a sucrose/proton antiport in the seed-coat membrane of *Phaseolus vulgaris.* The apparent link of sucrose uptake with corresponding H^+ export has been reported for the vacuolar membrane of the red beet root (Getz & Klein, 1995). It is unclear whether

similar mechanisms may exist in the plasmalemma of root cells. This question needs further investigation.

The possibility of metabolic regulation of oscillatory behaviour should also be considered. There are two major classes of cellular oscillators, membrane and cytoplasmic, and one component which is common to both of them is calcium (Berridge & Rapp, 1979). As the cytosolic calcium concentration, $[Ca^{2+}]_{cyt}$, is $<10^{-7}$ M (Evans, Briars & Williams, 1991), the Ca^{2+} influx caused by hyperosmotic treatment may lead to a significant rise in $[Ca^{2+}]_{cyt}$. Acting as a second messenger, Ca^{2+} may trigger a cascade of different metabolic events (Bush, 1993, 1995), modifying H^+ transport across the plasma membrane. McAinsh et al. (1995) reported stimulus-induced oscillations in *Commelina* guard cell cytosolic free calcium encoding information about the external stimuli. Does the same mechanism exist in the roots?

The last question concerns the time scale of increased $[Ca^{2+}]_{cyt}$. In hyperosmotic conditions the Ca^{2+} flux continued for at least 2 hr at an average of 8 to 10 nmol m^{-2} sec^{-1} (Fig. 2 and Table 1). Is all cytosolic Ca^{2+} used for intracellular signalling, or could it be effectively accumulated in the vacuole as an osmoticum, at least partially? We were not able to find in the literature any evidence of such osmotic adjustment for root cells, although during leaf ageing Ca^{2+} may accumulate preferentially in interstomatal cells at concentrations up to 180 mM (Fricke et al., 1995). Because we grew roots and measured fluxes in a low-salt nutrient solution containing only $CaSO_4$, these could be the only available inorganic ions for the osmotic adjustment. Further experiments are clearly needed. It will be reasonable also to assume that at least part of the absorbed Ca^{2+} would be exported to the shoot or to other root zones. Even in the control, Ca^{2+} concentration in the bath was normally 20 to 30 μM lower after 1–1.5 hr of plant incubation. This depletion of the Ca^{2+} indicates it was used for metabolic purposes or exported elsewhere in the plant.

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