3 Non-Invasive Microelectrode Ion Flux Measurements In Plant Stress Physiology

Sergey Shabala

3.1 Introduction: membranes and plant stress responses

Plant membranes underlie many essential cell biological processes including nutrient acquisition and compartmentation, pH and ionic homeostasis, turgor generation, metabolite distribution and waste excretion, energy transduction and signaling. According to Ward (2001), 43% of over 25,000 protein sequences in the *Arabidopsis* genome have at least one transmembrane spanning (TMS) domain, with 18% proteins having ≥ 2 TMS domains and thus associated with cellular membranes. Recent progress in electrophysiology and molecular genetics has revealed the crucial role of plasma membrane transporters in perception and signaling in response to virtually every known environmental factor (Zimmermann et al. 1999). Changes in plasma membrane potential or modulation of ion flux are amongst the earliest cellular events in response to light, temperature, osmotic stress, salinity, hormonal stimuli, elicitors and mechanical stimulation in many organisms (Blumwald et al. 1998; Sanders et al. 1999; Zimmermann et al. 1999; Spalding 2000; Knight and Knight 2001). For many, if not all the stresses mentioned above, the receptors involved were suggested to be located at one of the cellular membranes.

In addition to hosting various receptors mediating plant–environment interactions, membrane transporters always act as the ultimate effectors, enabling plant adaptive responses. In the case of salt tolerance, this may be by excluding toxic Na^+ from the cytosol via either the SOS1 plasma membrane Na^+/H^+ antiporter (Zhu 2003) or by compartmentalizing it into the vacuole by the NHX tonoplast Na^+/H^+ antiporter (Apse et al. 1999). In the case of Al^{3+} toxicity, the adaptive response includes activation of anion channels responsible for malate efflux and changes in the rhizosphere pH (Ryan et al. 2001). Osmotic adjustment includes rapid increase in the uptake of inorganic ions (Shabala and Lew 2002), while plant adaptive responses to low temperature include dramatic changes in membrane fluidity (Murata and Los 1997). Such a central role of plant membranes and membrane transport processes in plant adaptive responses to environmental conditions makes them important targets for genetic manipulations aimed to improve tolerance to a particular

School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia (e-mail: Sergey.Shabala@utas.edu.au)

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stress. To enable this, causal links between membrane-transport processes and other metabolic or physiological processes in the cell need to be understood.

Gaining such an understanding is not an easy task. It is complicated not only by the large number of transporters involved (for cations, 46 unique families are known, containing approximately 880 members in *Arabidopsis*; Maser et al. 2001), but also by the myriad of interactions and cross-talk between various transporters and signaling components. Over the last 2 decades, various state-of-the-art molecular and biophysical techniques (such as patch-clamp or fluorescence imaging) have been used to reveal some of these interactions. These techniques have been the subject of many comprehensive reviews and thus are mentioned only briefly here. However, at the same time, the inevitable consequence of such "in-depth" approaches was a decrease in the physiological reality of the transporters' environment (Tester 1997). There are many reports (some of which are discussed in section 4) showing that activity of a particular transporter differs dramatically when expressed in a heterologous system compared with in planta conditions. This makes it very difficult (and often even impossible) to transfer the results obtained by these advanced techniques to *real* plants in their natural habitats. The more advanced our study, the bigger is the gap between physiologists/molecular biologists and the agronomists interested in plant behavior in the field.

Since the mid-1990s our laboratory has pioneered application of non-invasive ion flux measuring (the MIFE) technique in plant stress physiology. As shown in the following sections, this technique provides a unique possibility to link genetic/genomic data to cellular physiological behavior. Some of its key features (e.g. non-invasiveness, high spatial and temporal resolution) allow us to establish and quantify causal links between membrane-transport processes and other metabolic or physiological processes in the cell in almost natural conditions. In this context, the MIFE technique may be considered as a "bridging element" between molecular biologists and whole plant physiologists or agronomists.

The aim of this review is to show that in situ measurements of net ion fluxes from plant cells and tissues using the MIFE technique can provide insights into the functional genomics of plants and will significantly increase our understanding of the function of specific genes mediating plant adaptive responses to the environment.

3.2 Basic techniques for studying membrane transport in plants

3.2.1 Comparative analysis of basic techniques

A large number of techniques are available to study ionic relations and transport of nutrients and ions across cell membranes. They range from whole-plant methods (depletion experiments, radioactive tracers) to those applicable

at cellular (fluorescence microscopy; intracellular microelectrode measurements) or molecular (patch-clamp studies on single channels) levels. Their pros and cons are briefly summarized below.

1. *Destructive sampling.* At the whole-plant level, basic chemical analysis of the elemental content in plant samples is still the most popular and widely used method. An appropriate plant sample is collected, dried, ground and digested in a strong acid (Handson and Shelley 1993). Then the elemental content of plant sample is analyzed using an appropriate technique (such as ICP-AES or AA spectroscopy; X-ray fluorescence spectroscopy, flame photometry). The advantage of this method is its simplicity. The major problem is a very low time resolution (usually several days or more), as there is an obvious limit on how often the samples may be taken. As a result, this method is mostly used to address some basic agronomical issues, rather than for fundamental physiological research.
2. *Depletion experiments.* This is another basic method used for many decades for studies on plant nutrition. Plants are grown hydroponically, and the rate of nutrient uptake is determined by periodically taking small volumes of the growth solution for chemical analysis (as above). Once again, the method has very low time resolution. The latter may be partially resolved by using conventional ion-selective electrodes placed in the growth solution to monitor concentration changes. Even then, however, the sensitivity of the method is rather low.
3. *Radioactive tracers.* Various radioisotopes have been successfully used to study membrane-transport processes in plants (Abbott and Fraley 1991; Tester and Davenport 2003). The method is relatively straightforward and allows quantification of the unidirectional flux of a specific ion. The main limitation is the spatial resolution of the method, as well as a relatively limited number of ions which can be studied.
4. *Nuclear magnetic resonance spectroscopy.* NMR spectroscopy is a non-destructive tool enabling quantitative analysis of metabolites from cell suspensions, tissues and whole plants (Ratcliffe 1997). As NMR detects atoms with magnetic moments only, not every nutrient can be studied. Taking nitrogen metabolism as an example, ^{14}N is naturally abundant (99.6%) but not useful for NMR studies due to extremely broad signals for almost all nitrogenous metabolites (Mesnard and Ratcliffe 2005). Therefore, plant samples have to be enriched with ^{15}N before analysis, which significantly complicates the procedure. Again, a low time resolution (several hours) is a problem.
5. *Fluorescence microscopy.* Ion imaging by fluorescence microscopy is based on fluorescence probes that accumulate inside cells and change their fluorescence properties when bound to distinct ions (Roos 2000). There is a relatively wide range of commercially available probes (both ratiometric and single-wavelength), enabling quantification and kinetic studies of changes in H^+, Ca^{2+} and, to lesser extent, K^+ and Mg^{2+} in plant cells. The method has high temporal and spatial (especially when confocal microscopy is used)

resolution and, being non-invasive, has a great potential for studying cellular adaptive responses to the environment. Major pitfalls include probe loading, photobleaching, interaction of ion probes with cell metabolism, difficulties of calibration, poor discrimination of some probes (e.g. between K^+ and Na^+) and the limited range of ions that can be measured by this technique (Roos 2000).

6. *Single cell sampling.* This technique has been developed by D. Tomos and co-workers as an extension of pressure-probe measurements at the single cell level (Tomos and Leigh 1999). By using a fine oil-filled glass microcapillary mounted on a micromanipulator, the cell sap is sampled from individual plant cells. Ion concentrations in these samples can then be analyzed using a range of physical and chemical methods such as X-ray microdroplet analysis or capillary electrophoresis (Tomos and Leigh 1999). The method has very high spatial resolution. Two main obstacles limiting its application are (i) the issue of mixing of vacuolar and cytosolic content and (ii) impossibility of kinetics study by this method.
7. *Patch-clamp.* The patch-clamp technique is the most advanced method of studying ion-transport processes at the molecular level (Tester 1997) and can provide comprehensive information about the kinetics and properties of specific transport proteins at cell membranes. The method is based on a tight attachment of a plasma membrane patch to a microelectrode glass pipette, thus establishing a so-called "giga seal" (with up to 10^9 Ω resistance), enabling measurements of very low (pA range) currents through the prepared isolated plasma membrane patch in response to a series of voltage clamps. Ultimately, membrane ion channels may be characterized with respect to the ion they conduct (and their specificity for that ion), the conductance value of their open state, their gating properties, and their sensitivity to various pharmacological agents (Garrill and Davies 1994). However, patch clamp is rather sophisticated method that requires high level technical and data interpretation skills. Quite often, conditions that enable seal formation are rather non-physiological (e.g. high amount of Ca^{2+} in the pipette). As a result, it is not always possible to extrapolate patch-clamp data onto ion channel behavior in *planta*.
8. *Impaled microelectrodes.* Traditionally, microelectrode impalement has been applied to measure plasma membrane potential (MP). Significant membrane depolarization is observed in response to various environmental stresses such as salinity (Shabala et al. 2003), chilling (Clarkson et al. 1988), acidity (Babourina et al. 2001) and hypoxia (Zhang et al. 1992). As transport of all nutrients is directly or indirectly linked to MP values, the more substantial is the membrane depolarization, the more severe is the disturbance to cell ionic homeostasis. A more sophisticated method involves a microelectrode tip being filled with a specific ionophore, sensitive to a particular ion (Miller et al. 2001). As the reference MP electrode also has to be impaled alongside the ion-selective microelectrode, multibarreled electrodes are often used for these purposes (Carden et al. 2003).

This technique thus makes it possible to monitor changes in cytosolic ion homeostasis and therefore provide answers to some fundamental questions about ionic mechanisms underlying stress tolerance in plant cells. However, this technique is extremely technically and skill demanding, and thus not likely to be used routinely.

3.2.2 Non-invasive ion flux measurements

In recent years, non-invasive microelectrode ion flux measurements have become a popular tool in studying adaptive responses of plant cells and tissues to a large number of abiotic stresses. A couple of dozen laboratories around the world employ this technique, and their number is growing.

The idea of using slowly vibrating ion-selective microprobe to measure non-invasively net ion fluxes was first proposed by B. Lucas (Lucas and Kochian 1986). The microelectrodes were proposed to measure ion concentration *gradients* (strictly, electrochemical potential differences), between two positions in solution outside the organism tissues, and to use those gradients to calculate the *net fluxes* of ions in question crossing the membrane. The first rigorous test of the theory was performed on corn roots to measure the stoichiometry of H^+ and K^+ fluxes (Newman et al. 1987). The National Vibrating Probe Facility at Woods Hole, Mass., USA adapted the vibrating probe to include ion flux measuring capability (Smith 1995). An alternative system, named MIFE (*M*icroelectrode *I*on *F*lux *E*stimation), was developed, at about the same time, by I. Newman at the University of Tasmania in Australia (Newman 2001). Since the mid 1990s, MIFE has been successfully applied to the study of various aspects of membrane-transport processes in plants and protoplasts derived from plant tissue (Newman's and Shabala's groups) resulting in nearly 50 publications. Recently, the MIFE technique was successfully used to measure kinetics of ion fluxes from bacterial membranes (Shabala et al. 2001a). Several alternative systems have been also designed elsewhere (e.g. Shipley and Feijo 1999); some of them being commercially available. Several papers in this book provide further specific details on some of these systems.

There are at least five major features that, taken together, provide a significant advantage of the MIFE approach over other methods for ion flux measurements. These include:

1. *Non-destructiveness.* In contrast to many other methods, the MIFE (or other similar) technique allows in-situ measurements of net ion fluxes, in physiologically "realistic" conditions.
2. *High spatial resolution.* The electrode tip is several (typically 2–3) μm in diameter, which makes it possible to measure net ion fluxes from single cells (Babourina et al. 2000; Shabala et al. 2001b) or protoplasts derived from plant cells (Shabala et al. 1998; Tyerman et al. 2001). Moreover, for some ionophores with high signal-to-noise ratio (such as H^+), the electrode

tip diameter can be further reduced to 0.8–1.0 μm. As a result, the cell surface can be "mapped" (Shabala et al. 1998; Tegg et al. 2005), providing information about spatial distribution and functional expression of specific ion transporters.

3. *High temporal resolution.* The "default" MIFE settings assume electrode movement with 10 s period. This could be further reduced without difficulty to 2 or 3 s in some cases. Such high temporal resolution is especially crucial in studying rapid signaling events at plant membranes. Most other non-invasive techniques operate on a time scale at least 1 order of magnitude slower. This gives the MIFE technique a unique opportunity to provide insights into very early (and fast) events associated with plant responses to environmental changes.
4. *Duration of measurements.* As the technique is non-invasive, its application is limited essentially only by the lifetime of the ion-selective electrode (typically 15–20 h). Moreover, electrodes may be easily replaced, and measurements resumed after only a few minutes break. None of the other techniques of the same time resolution (e.g. patch-clamp or fluorescence imaging) provide this opportunity.
5. *Simultaneous measurements of several ions.* The possibility of measuring kinetics of fluxes of several ions simultaneously, and essentially at the same spot, is important in understanding the underlying ionic mechanisms of cell adaptive responses. By assessing stoichiometry ratios between various ions, valuable information about the membrane transporters involved can be gained.

3.3 MIFE technique for non-invasive ion flux measurements

3.3.1 Theory

The theory of non-invasive MIFE ion flux measurements was reviewed in detail by Newman (2001). In this section, I will only reiterate some of its basic principles.

Briefly, if an ion is taken up by living cells, its concentration in the proximity of the cell surface will be lower than that further away. Vice versa, if the ion is extruded across the plasma membrane, there will be a pronounced electrochemical potential gradient directed away from the cell or tissue surface (Fig. 3.1). The principle of the MIFE technique is in measuring this electrochemical potential gradient by slow square-wave movement of ion-selective electrode probes between two positions, close to (position 1), and distant from (position 2) the sample surface (Fig. 3.1). At each position, electrode voltage is recorded and then converted into approximate concentration using the calibrated Nernst slope of the electrode. It is assumed that convection and water uptake are negligibly small and unstirred layer conditions are met (Newman 2001).

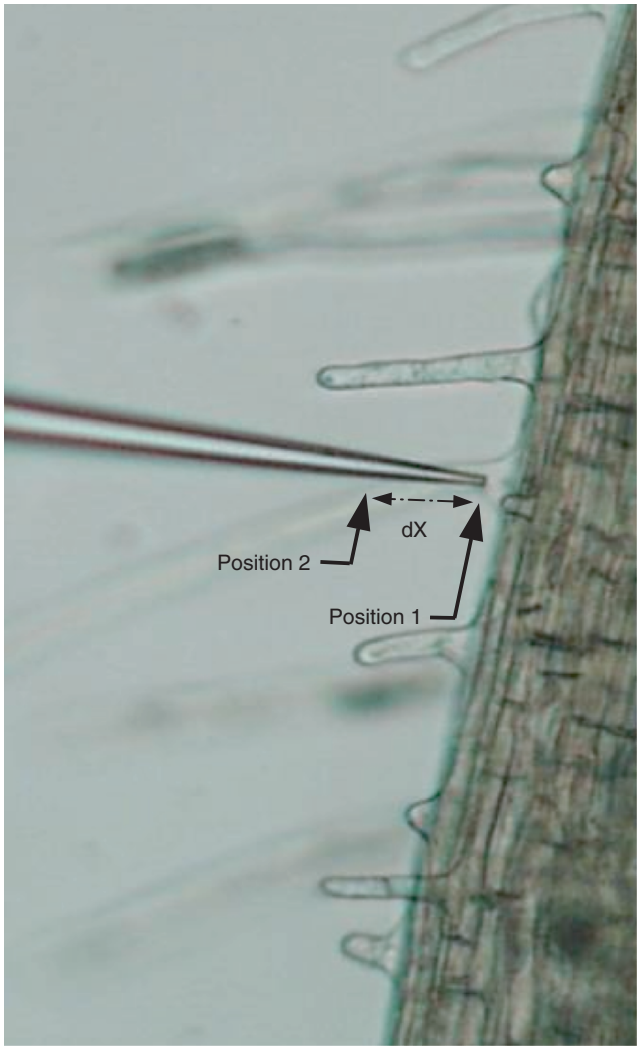

Fig. 3.1. Basic principles of the MIFE ion flux measurement. The ion-selective microelectrode is moved in a square-wave manner between two positions near the root surface. A voltage gradient (dV) is measured by the electrometer between two positions over the travel range dX

Net fluxes of specific ions (mmol m^{-2} s^{-1}) can then be calculated from the measured voltage gradient near the surface. The magnitude of the flux is strongly dependent on the tissue geometry, determining ion diffusion profiles. In the simplest case of planar diffusion (such as from plain leaf surface), the following equation is used (Newman 2001):

$$J = cuz\,Fg\,(dV/dx),$$

where c is ion concentration (mol m^{-3}); u is the ion mobility (m s^{-1} per Newton mol^{-1}); z is the ion's valence; F is the Faraday number (96,500 °C mol^{-1});

g is a factor found from the measured Nernst slope for the electrode during calibration; dV is the voltage difference measured by the electrometer between the two positions (V); dx is the distance between two positions (m).

For cylindrical geometry (e.g. root surface) the radius of the cylinder (r) should be taken into account. This is done by replacing dx in the implementation of the equation above by

$$dx = r^2\left[1/(r + x) - 1/(r + x + dx)\right].$$

For spherical geometry (e.g. protoplast)

$$dx = r\ln\left[(r + x + dx)/(r + x)\right].$$

3.3.2 MIFE hardware

The MIFE setup is built around the microscope system with long distance objectives. There are several basic configurations of the MIFE system used in our laboratory. When fluxes are to be measured from small specimens (such as single cells; protoplasts; bacterial monolayers), an inverted microscope is used. The ion-selective electrodes are mounted on a multi-manipulator providing 3-dimensional fine positioning of the electrode tips near the specimen surface. The manipulator is attached to the stepper motor-driven 3-dimensional hydraulic micromanipulator, enabling the square-wave electrode movement to measure the electrochemical potential of the ions at two positions in solution close to a tissue surface.

The measured specimen is immobilized at the bottom of the open type experimental chamber. The chamber is placed on the microscope stand, and electrode holders are positioned at an angle of 30° to the microscope stand plane. The standard non-polarizing Ag/AgCl reference electrode is positioned in the chamber. The electrodes oscillate (usually at 0.1 Hz), between two positions, close (usually 10–20 μm) and more distant (40–50 μm) from the cells, driven by the computer-controlled stepper motor. The voltage output from the electrodes is amplified by the MIFE electrometer and digitized using an analogue-to-digital interface card on an IBM-compatible PC. The card also controls the stepper motor of the manipulator and is used for offset adjustment of the four-channel electrometer.

The DOS-operated CHART (University of Tasmania, Hobart, Australia) software allows automated and interactive real-time control of the amplifier configuration and the micromanipulator while the data is being collected and written to disk (Shabala et al. 1997). The recorded voltage traces are displayed on the screen in a real time scale (Fig. 3.2), with a possibility of expanding or contracting some selected data segment without interrupting the measurements. More details are available at http://www.mife.com.

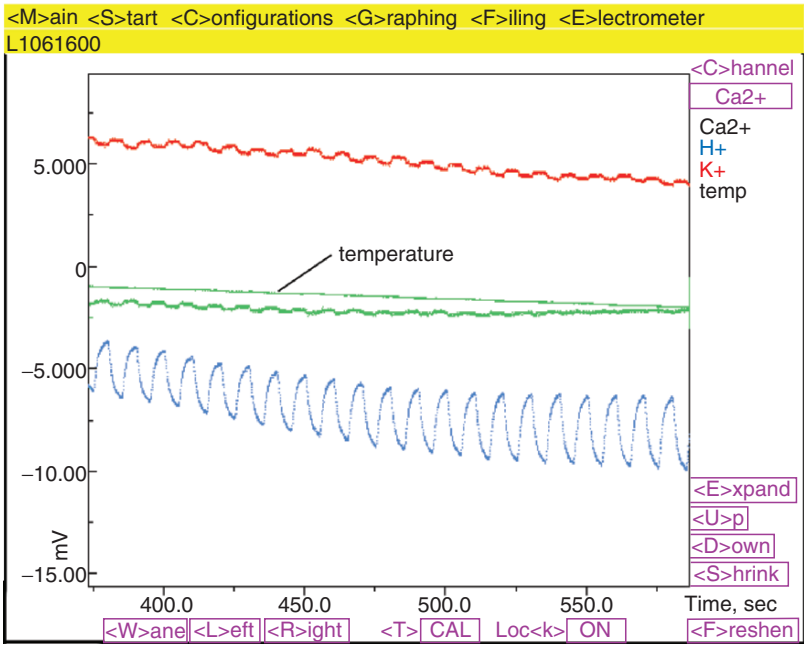

Fig. 3.2. Computer screen display of the MIFE recordings (courtesy Dr. L.Shabala). A (reverse colour) screen is pictured showing four concurrent voltage records for Ca^{2+}, H^+, K^+, and temperature. Fluxes of ions were calculated from the voltage (ΔV) using recorded concentration values. H^+ and K^+ concentrations are decreasing slowly. K^+ and H^+ fluxes are steady, while Ca^{2+} flux decreases to zero. Temperature (reversed scale) increases slowly

Flux calculations are performed automatically by the MIFEFLUX software (University of Tasmania, Hobart, Australia) from the data and log files. Calculated ion fluxes (in nmol m^{-2} s^{-1}) and concentrations are exported into an ASCII-format spreadsheet and saved onto computer disk, alongside the raw (mV records) data.

For ion flux measurements from root or leaf surface, a more "user-friendly" arrangement is used. In this case, the MIFE setup is built around the standard stereomicroscope. The microscope is rotated by 90°, so that the optical axis is horizontal. The measuring chamber is mounted on the computer-driven 3-dimensional hydraulics micromanipulator, while microelectrodes are held by the MMT-5 manipulator. Under such an arrangement, the electrodes are steady, and it is a slow movement of the measuring chamber that enables flux measurement. The main advantage of this arrangement is the convenience of electrode positioning (much further from the chamber's bottom—thus, less danger for electrodes to be broken). For more details, refer to Shabala and Newman (1997).

3.3.3 Methodological issues

Liquid membrane type ion-selective microelectrodes are used for MIFE measurements. Specific details of their fabrication and calibration are given in our publications (e.g. Shabala et al. 1997, 2005a; Shabala and Shabala 2002). Briefly, electrodes are pulled from non-filamentous borosilicate glass capillaries to tip diameter ~1 μm. Electrode blanks are then silanized with tributylchlorosilane (90796; Fluka Chemicals) to make their surface hydrophobic. Dried and cooled electrode blanks are stored under cover and may be used over several weeks. To make the electrode, the electrode tip is first broken slightly to achieve the required diameter (typically 2–3 μm). It is then backfilled with an appropriate solution (see Shabala and Shabala 2002 for more details) and finally front-filled with the appropriate liquid ion exchanger (LIX). Immediately after filling, electrodes are immersed in solution and kept there until use (up to 8–10 h).

In our laboratory, we routinely use the MIFE system to measure net fluxes of H^+, Ca^{2+}, K^+, Na^+, Cl^-, Mg^{2+}, NH_4^+, NO_3^-, and Cd^{2+} from various systems: higher plant tissues and protoplasts; animal tissues (e.g. muscles); fungi; algae; protists; yeasts; bacterial monolayers and biofilms. Several more ions (e.g. Zn^{2+}, Cu^{2+}, Cs^+, Pb^{3+}, SO_4^{-2}) also can be measured using commercially available ionophores.

Despite the same principle being used for measurements of fluxes of each of these ions, there are some "specific features", related to fabrication, calibration, and use of ion-selective microelectrodes to measure fluxes of a specific ion. The full coverage of these issues is beyond the scope of this paper. Some of these issues are also covered in detail in previous reviews on MIFE (Newman 2001; Shabala et al. 2005a). In this paper, only the crucial issues are mentioned.

1. *Basic electrode characteristics.* As a rule, for most physiological conditions, electrode characteristics are expected to be linear. Accordingly, each electrode is calibrated in a set of three known standards, covering the range of concentrations expected to be found in the experiment. The average responses of electrodes are about 53–54 mV/decade for monovalent ions, and 27–28 mV/decade for divalent ions, with a correlation R>0.999. If measurements are made at very low concentrations, non-linearity requires that more than 3 standards should be used.
2. *Electrode "conditioning".* Most of the prepared microelectrodes can be used immediately after preparation, while others (e.g. H^+ and Cl^-) need some conditioning time (~1 h) to ensure a stable response.
3. *Responsiveness.* During MIFE measurements, the electrodes are moved back and forth at 5-s intervals. For accurate flux calculations, the LIX must "settle" at each position. From practical experience, settling achieved quicker if the LIX column length is relatively short. However, in this case there is a danger of a gradual leak of LIX out of the tip and electrode loosing its

selectivity. The compromise is achieved by optimizing the amount of silane used for electrode fabrication and the amount of LIX used for electrode filling.

4. *Effect of ionic strength.* Variations in ionic strength of solutions might significantly affect characteristics of ion selective electrodes and result in inaccurate estimates of ionic concentrations and, ultimately, net ion fluxes. The actual concentration (and, thus, flux) is overestimated for solutions with ionic strength lower than that of the standard, and is underestimated vice versa. For many ions, such a difference may be as big as a factor of 2 in a physiologically relevant range of concentrations (e.g. Na^+ levels 200 mM). More details are available in Shabala et al. (2005a).
5. *Temperature.* Although the theory shows that the Nernst slope is proportional to the Kelvin temperature. However, our experiments in the 4–40 °C range suggested that in all cases, the Nernst slope remained > 50 mV/decade, and the maximum inaccuracy in flux calculation did not exceed 6% (Shabala et al. 2005a). Thus, for practical purposes, the effects of temperature on electrodes may be ignored. However at temperatures above 32 °C, the LIX often became very "noisy" affecting the signal to noise ratio. This should be kept in mind when planning experiments.
6. *Signal to noise ratio.* Due to the thermal electron noise in electrodes, there is some theoretical "lower limit" on the magnitude of the flux that can be measured against the background noise (Ryan et al. 1990). There are two practical measures to overcome this problem and to improve the sensitivity of the flux measurements. One is to increase the travel range of the electrode (making voltage changes larger), and another one is to increase electrode tip diameter. For more details, see Shabala et al. (2005a).
7. *Confounding effect of inhibitors.* Pharmacological experiments are frequent in plant electrophysiology. However, many of the channel blockers and metabolic inhibitors routinely used in patch-clamp experiments may significantly affect LIX characteristics. For example, even micromolar concentrations of CCCP completely "killed" Ca^{2+} LIX, reducing electrode slope from 27 to < 3 mV/decade. Therefore, a rigorous test of LIX performance in the presence of inhibitors should be undertaken first.

3.4 Application of ion-selective microelectrodes to study plant adaptive responses to environmental conditions

3.4.1 Nutritional disorders

A large bulk of literature is available dealing with the application of ion selective microelectrodes to study various aspects of nitrogen (both NH_4^+ and NO_3^-; Henriksen et al. 1990, 1992; Colmer and Bloom 1998; Garnett et al. 2001, 2003) and potassium (Newman et al. 1987; Ryan et al. 1990; Jones et al. 1995) nutrition

and transport in plant roots. These studies revealed a complex heterogenous nature of nutrient acquisition along the root axis, with some pronounced differences between functional root regions. Most of these issues have been comprehensively reviewed by Newman (2001), so are not discussed here. The only aspect I would like to comment on here is the possibility of measuring ion fluxes from root hairs. Root hair growth appears to be linked intimately to the cytosolic free Ca^{2+} concentrations in the apex (Gilroy and Jones 2000). High spatial resolution of the microelectrode ion flux measuring technique allowed fine-scale quantification ("mapping") of ion fluxes from the growing root hair (Jones et al. 1995). These authors reported tip-localized influx of H^+ and Ca^{2+}, with relatively uniform K^+ influx along the length of the root hair.

The ion flux measuring technique is also widely applicable for the study of mechanisms of uptake and translocation of micronutrients in various plant tissues. Studies in Kochian's lab showed the potential for the application of Cd^{2+}-selective microelectrodes to study heavy-metal ion transport in roots (Pineros et al. 1998). Several more LIX are commercially available from Fluka (e.g. Cu^{2+}, Zn^{2+}, Pb^{2+}, Cs^+). Being combined with molecular tools, their use may greatly advance engineering of varieties with higher nutrient efficiency, as well as helping in phytoremediation of metal-contaminated soils.

3.4.2 Salinity

Salt tolerance in non-halophytes is conferred by a large number of adaptive mechanisms, most of which are related to membrane-transport processes. Recently, this topic has been the subject of a large number of comprehensive reviews (e.g. Maathuis and Amtmann 1999; Hasegawa et al. 2000; Zhu 2002, 2003; Tester and Davenport 2003). Also, molecular and ionic mechanisms involved in the regulation of K^+ homeostasis and maintaining an optimal K^+/Na^+ ratio (critical to salt tolerance) in salinized plant tissues are discussed in detail in another review by Cuin and Shabala in this book. Here, I want to discuss only a few specific aspects of the application of the MIFE technique to study mechanisms of salt tolerance in plants.

3.4.2.1 Specific and non-specific components of salt stress

Two principal adverse effects of salinity in non-tolerant plants are osmotic stress and specific ion (Na^+ or Cl^-) toxicity (Munns 2002; Zhu 2003; Tester and Davenport 2003). The intriguing question is: how do plants distinguish between these two?

The above issue was addressed by measuring net ion flux responses to isotonic NaCl and mannitol solutions from various leaf (Shabala 2000) and root (Chen et al. 2005) tissues. While NaCl promoted a net K^+ efflux, isotonic mannitol treatment induced a gradual increase in the net K^+ uptake from both leaf and root tissues, indicating that different ionic mechanisms are

involved in perception of "ionic" and "osmotic" components of the salt stress. Pharmacological and patch-clamp experiments suggested that NaCl-induced K^+ efflux is mediated essentially by depolarization-activated K^+ outward-rectifying channels (DAPC), while inward-rectifying K^+ channels are involved in K^+ uptake in response to mannitol treatment. Overall, our results showed that K^+ fluxes from plant cells under salt conditions are driven by two oppositely directed signals: (i) K^+ efflux resulting from NaCl-induced plasma membrane depolarization and (ii) K^+ uptake resulting from some elusive "osmosensing mechanism" (see section 4.3 for details). Under mild salinities, the latter component dominates, while higher NaCl treatments result in overall net K^+ efflux from salinized tissues.

3.4.2.2 Delineating the role of the plasma membrane H^+-pump in salt stress responses

The other important issue concerns the involvement of the plasma membrane ATP-dependent electrogenic H^+-pump in cellular responses to salt stress. A NaCl-induced increase in plasma membrane H^+-ATPase activity has been reported for many halophytic species (Ayala et al. 1996; Vera-Estrella et al. 1999). It was suggested that the stimulation of H^+-ATPases by salt stress may provide a driving force for a plasma membrane Na^+/H^+ exchanger to move Na^+ from the cytoplasm into the apoplast, thereby providing a significant contribution to the salt adaptation of plant cells (Ayala et al. 1996). However, Serrano et al. (1999b) concluded that cells confronted with toxic cations such as Na^+ temporarily down-regulate their H^+-pump to escape stress. To shed more light on this issue, H^+ fluxes were measured from the mesophyll tissue of salt-sensitive broad bean species (Shabala 2000) in response to NaCl treatment. Our results showed a significant increase in NaCl-induced net H^+ efflux. Leaf pre-treatment with CCCP (a protonophore) and orthovanadate (a specific inhibitor of the plasma membrane H^+ pump) did not prevent the initial "instantaneous drop" towards net H^+ efflux, but did completely arrest the subsequent continuous drift towards larger efflux (Shabala 2000). It was concluded that there are at least two components of the observed H^+ flux: one is "vanadate-sensitive" (suppressed by both vanadate and CCCP), and another is "vanadate-insensitive". Their origin is a matter for further investigation.

3.4.2.3 K^+ homeostasis as a key feature of salt tolerance

The ability of a plant to prevent accumulation of excessive amounts of Na^+ in the cytosol has always been considered as a key feature of salt tolerance (Munns 2002; Zhu 2003; Tester and Davenport 2003). This may be achieved either by preventing Na^+ uptake, or by active Na^+ extrusion from cytosol into the apoplast (via plasma membrane SOS1 Na^+/H^+ antiporter) or into the

vacuole (via tonoplast NHX antiporter) (Blumwald et al. 2000; Hasegawa et al. 2000; Tester and Davenport 2003; Zhu 2003). Accordingly, plant breeding for salt tolerance was traditionally aimed to improve one of those features (Blumwald et al. 2000; Munns 2002; Zhu 2003). However, it appears that it is K^+/Na^+ *ratio* in the cytosol rather than Na^+ concentration per se that is critical to salt tolerance (Maathuis and Amtmann 1999). Direct evidence for this is surprisingly rare (Carden et al. 2003), most likely due to technical difficulties associated with measuring cytosolic Na^+ and K^+ concentrations.

Use of the MIFE technique offers an excellent opportunity to look at mechanisms underlying K^+ homeostasis in salinized plant tissues. Recently, we have made a rigorous comparison of physiological responses of seven barley cultivars contrasting in their salt tolerance (Chen et al. 2005). A very strong correlation ($R>0.8$) was shown to exist between net K^+ fluxes measured from the surface of 3-day-old barley roots after 40 min of treatment in various concentrations of NaCl and plant physiological responses (growth rate, biomass, net CO_2 assimilation, chlorophyll fluorescence, root and leaf elemental and water content) after a month of NaCl treatment in a glasshouse. The difference between NaCl-induced K^+ efflux between tolerant and sensitive cultivars was 3-fold, with much higher K^+ efflux measured from sensitive varieties (Chen et al. 2005). Importantly, this feature was heritable, as shown in experiments on F1 and F2 back-crosses. These findings not only suggest that K^+ homeostasis is a key feature for plant salt tolerance, but also offer a convenient non-destructive screening tool for plant breeders.

3.4.2.4 Ameliorative effects of Ca^{2+}

It is also known that the application of external Ca^{2+} may significantly ameliorate salinity stress symptoms in many species (Cramer et al. 1987; Rengel 1992; Reid and Smith 2000). It was traditionally believed that the dominating mechanism of such amelioration was in Ca^{2+} restriction of Na^+ uptake via non-selective cation channels (NSCC) which are believed to be a major pathway for Na^+ uptake into the cell under conditions of high salinity (Tyerman et al. 1997; Demidchik and Tester 2002; Tester and Davenport 2003). Recent studies provided some evidence that not only Ca^{2+} but also other divalent cations may be important for controlling Na^+ transport across the plasma membrane in saline conditions (Elphick et al. 2001; Demidchik and Tester 2002). Until recently, it remained unclear whether NSCC blockage by elevated $[Ca^{2+}]$ was the only mechanism involved. MIFE experiments on both root (Shabala et al. 2003) and leaf (Shabala 2000; Shabala et al. 2005b) tissues of various species showed that supplemental Ca^{2+} efficiently reduces, or even completely prevents, NaCl-induced K^+ efflux from the cell. Other divalent cations (Mg^{2+}, Ba^{2+}, Zn^{2+}) were also efficient in preventing K^+ leakage in response to salinity (Shabala et al. 2005b). Patch-clamp experiments on *Arabidopsis* leaf and root cells implicated DAPC involvement (S. Shabala, V. Demidchik and J. Davies, submitted).

Overall, our results suggest that, in addition to their known ability to block NSCC, divalent cations also control the activity or gating properties of K^+ transporters at the cell plasma membrane, thereby assisting to maintain the high K^+/Na^+ ratio required for optimal plant growth and leaf photosynthesis.

3.4.3 Osmotic stress

Improving crop resistance to osmotic stresses has always been a focus of breeding programs. Over the last 2 decades, the major emphasis was on molecular engineering of transgenic species, which overexpress genes responsible for biosynthesis of various compatible solutes (Bohnert et al. 1995; Bajaj et al. 1999; Bohnert and Shen 1999; Serrano et al. 1999a). It is traditionally believed that de novo synthesis of such compatible solutes is involved in re-adjustment of cell osmotic potential and prevention of water losses. However, it recently became evident that the functions of compatible solutes are not likely to be limited to conventional osmoprotection. Instead, a regulatory role for compatible solutes in adjustment of metabolic pathways to altered environmental conditions, was postulated (Bohnert and Sheveleva 1998; Serrano et al. 1999b). An important component of such adjustment might be control over activity of membrane ion transporters also involved in cell osmotic adjustment.

It has long been suggested that changes in ion fluxes in response to osmotic stress provide quick (within a few minutes) osmotic adjustment and maintain normal turgor (Wyn Jones and Pritchard 1989; Bohnert et al. 1995; Lew 1996). However, until recently, no direct evidence was provided. Using a non-invasive ion flux measuring technique, we showed that bean mesophyll cells responded to hyperosmotic stress by increased uptake of K^+ and Cl^- (Shabala et al. 2000). This is consistent with other reports obtained using different experimental techniques (Okazaki et al. 1984; Teodoro et al. 1998). Our model calculations estimated that up to 85 % of the changes in the cell turgor may be compensated by uptake of these two inorganic ions within 1 h after stress onset. These calculations were validated in direct concurrent measurements of net ion fluxes and cell turgor changes (the pressure-probe technique) in osmotically stressed *Arabidopsis thaliana* epidermal root cells (Shabala and Lew 2002). Our results showed that > 90% of the cell turgor was recovered by inorganic ion uptake within 40 min after onset of hyperosmotic stress.

There are at least two major advantages in using inorganic ions for cell osmotic adjustment. One of them is the rapidity of turgor recovery (minutes versus. hours or days for de novo synthesis of compatible solutes). This may give an adaptive advantage to plants which experience acute water stress in natural conditions throughout ontogeny (such as with a shallow root system). Another advantage is the energetics of osmotic adjustment. According to Raven (1985), the ATP requirements for synthesis of compatible solutes is approximately 10 times higher than that for active ion transport across membranes.

Specific ionic mechanisms involved in osmotic stress perception are still elusive. Lew (1996) suggested that *Arabidopsis* root hair cells possess an osmosensing but not a turgor-sensing mechanism. At least two mechanisms, namely (i) mechanosensitive (or stretch-activated) channels and (ii) putative intracellular osmosensing mechanisms, have been suggested by Brownlee et al. (1999). However, most reported evidence of SAC was obtained by using the patch-clamp technique; there is thus a need for more experimental observations of SAC effects at the tissue or organ level. Appearance of the additional 2-min oscillatory component in ion flux oscillations measured from hyperosmotically stressed plant roots (Shabala and Newman 1998) may provide such evidence.

A ubiquitous component of osmotic adjustment in higher plants is modulation of the proton-pumping activity (Reinhold et al. 1984; Li and Delrot 1987; Reuveni et al. 1987). Palmgren (1991) suggested that the relaxation of the stretched status of the membrane might directly activate the plasma membrane H^+-ATPase, as the activity of this enzyme is strictly dependent on the lipid environment. Passive H^+ transport is also affected (Bisson and Gutknecht 1977; Lew 1996) implying a multicomponent osmo-regulatory system. It remains to be answered whether the plasma membrane H^+-pump is a primary target (a receptor) of osmotic stress (Reinhold et al. 1984), or merely a component of the complex signaling network controlling the activity of the plasma membrane transporters for other ions (Kinraide and Wyse 1986; Li and Delrot 1987). Supporting evidence for the former hypothesis includes reports of significant osmotic-induced acidification of the bathing medium (Kinraide and Wyse 1986; Reuveni et al. 1987) and direct measurements of net H^+ extrusion (Lew 1998; Shabala and Newman 1998; Shabala et al. 2000).

At the same time, some authors ruled out direct effects of osmoticum on the H^+ pump, suggesting instead that the primary targets in the osmosensory mechanism are stretch-activated Cl^- channels inactivated by hyperosmotic stress (Teodoro et al. 1998; Zingarelli et al. 1999). In our experiments, K^+ uptake kinetics in hyperosmotically stressed mesophyll tissue was not affected when 500 μM vanadate (a known inhibitor of the H^+-ATPase) or 50 μM CCCP (a protonophore) were added (Shabala et al. 2000). At the same time, when K^+ uptake was suppressed by 20 mM TEA, neither K^+ nor H^+ flux changes were evident in response to mannitol treatment. This finding suggests that direct control of K^+ uptake is an important part of the process of osmoregulation. Earlier Liu and Luan (1998) suggested voltage-dependent K^+ and Cl^- channels being potential targets of osmosensing in stomatal guard cells. Direct modification of the turgor activated ionic conductance for K^+ and Cl^- was also shown in *Chara* (Kourie and Findlay 1991). The exact mechanism of this modulation, however, remains unclear.

Enhanced H^+ extrusion (Reinhold et al. 1984; Li and Delrot 1987; Reuveni et al. 1987) is expected to cause significant membrane hyperpolarization. This is indeed the case (Reid et al. 1984; Kinraide and Wyse 1986; Reuveni et al. 1987; Teodoro et al. 1998; Zingarelli et al. 1999; Shabala and Lew 2002). Such

hyperpolarization may enhance K^+ uptake via voltage-gated K^+ inward-rectifying channels or, alternatively, reduce K^+ efflux through outward K^+ channels (Lew 1996; Zingarelli et al. 1999). Both these types of channels are known to show strong voltage dependence (Very and Sentenac 2002; Shabala 2003), and the possibility that voltage clamp of the plasma membrane directly affects net K^+ fluxes into and out of the cell was shown in direct experiments combining the voltage-clamp and MIFE techniques (Shabala and Lew 2002).

3.4.4 Temperature extremes

Low ambient temperatures have a significant impact on agricultural production not only of many species of tropical or subtropical origin grown in temperate regions around the world, but also within a normal plant's habitat (Lyons 1973). Genetic manipulation of chilling-sensitive species for achieving better growth and higher crop yield depends on an understanding of mechanisms of chilling responses and tolerance.

There are numerous papers reporting significant perturbations in root ion uptake and transport under chilling stress (Bravo-F and Uribe 1981; White et al. 1987; Pritchard et al. 1990; Petterson 1995). Results are often controversial. Strong temperature dependence for uptake of potassium and phosphorous was reported for corn roots (Bravo-F and Uribe 1981), while Petterson (1995) found no effect of low root zone temperature on net uptake or transport of these ions in barley. Wang et al. (1993) have reported that high and low affinity transport systems for NH_4^+ in rice roots had very different Q_{10} (2.6 and 1.5, respectively). However, no attempts to directly measure the temperature dependence of fluxes of different nutrients in situ at the cellular level have been undertaken until recently.

Using the unique advantages of the MIFE technique, we showed that critical temperatures, under which the recovery of the activity of plasma membrane transporters took place, was the same for all ions measured (H^+, Ca^{2+}, K^+, Na^+, NH_4^+, and Cl^-; Shabala and Shabala 2002) and very close to the critical temperature of plasma membrane phase transition expected to be found for the measured species (Lyons 1973). We also showed, at the same time, that the apparent difference in ion flux kinetics measured in a traditional way may be misleading, due to different rates of recovery for different transporters (Shabala and Newman 1997).

The primary sites of the chilling effect appear to be plant membranes (Lyons 1973; DuPont 1989; Yoshida 1991). The role of membrane fluidity in temperature perception and transduction of the chilling signal now seems to be indisputable (Marschner 1995; Murata and Los 1997). Chilling temperatures may directly affect the structure and function of the plasma membrane transport enzymes. Changes in lipid composition, carrier activity, permeability, and ultrastructure have been shown to occur at the membrane level (Hällgren and Öquist 1990 and references within). However, specific mechanisms underlying

the effects of membrane phase transition on activities of plasma membrane transporters remain to be elucidated.

Several possibilities have been suggested, including conformational changes of transport proteins (changing affinity), or indirect effects due to modification of the physical properties of the lipid environment (Clarkson et al. 1988). As most membrane transporters are coupled via MP, conformational changes in one transport protein will most likely have a domino effect on the activity of others. Our experiments (Shabala and Shabala 2002) suggest that two transporters of ions are primary "suspects" for such a role.

Plasma membrane H^+-pump. There is much evidence in the literature that active H^+ transporters are among the most sensitive to temperature stress in chilling-sensitive species (DuPont 1989; Yoshida 1991). In our experiments, both vanadate and CCCP caused an 80% reduction in the magnitude of H^+ flux temperature responses (Shabala and Shabala 2002). Observed changes in the activity of other transporters could be explained by the dramatic depolarization of the plasma membrane (Clarkson et al. 1988). A resultant shift in the values for the Nernst potential would enhance the passive cation leak from the cells (Clarkson et al. 1988; Hällgren and Öquist 1990; Wang et al. 1993; Macduff and Dhanoa 1996; Shabala and Shabala 2002).

Cold induced inactivation of the H^+-ATPase may have been due to a structural alteration in the enzyme complex, including a modification of the intermolecular association of the subunits and the uncoupling of ATP hydrolysis and H^+ transport activity (Yoshida 1991). When the principal processes generating ATP temporarily collapse under chilling conditions, the ATP-driven efflux of H^+ will decrease (Clarkson et al. 1988). This is exactly what was observed when measuring net H^+ fluxes in chilled root and leaf tissues (Shabala and Newman 1997).

Ca^{2+} transporters may play a central role in mediation of low-temperature effects on cell nutrient uptake. All tested metabolic inhibitors (La^{3+}, vanadate and TEA) caused dramatic reduction (>90%) in the magnitude of Ca^{2+} flux responses to chilling recovery (Shabala and Shabala 2002). As some of these inhibitors are not Ca^{2+}-specific, it indicates a likely signaling role of Ca^{2+} flux changes (Knight et al. 1996; Murata and Los 1997). According to some models, Ca^{2+} channels open at low temperatures upon a decrease in membrane fluidity, and the entering Ca^{2+} ions activate a signal transduction pathway for up-regulation of the expression of low-temperature-inducible genes (Monroy and Dhindsa 1995). Validation of this model and elucidation of a mysterious putative sensor at the plasma membrane remains a priority for future research.

3.4.5 Soil pH

Soil acidity severely limits plant production worldwide. Among the major constraints, Al^{3+} toxicity is the most important (Marschner 1995), with even micromolar concentrations of Al^{3+} in the soil solution inhibiting the root

growth of many species (Kochian 1995; Ryan et al. 2001). The mechanisms of Al^{3+} toxicity are complex and may include interactions that occur both in the symplast and apoplast (Kochian 1995).

The ability of many species to maintain root growth at low soil pH is associated with the release of weak organic acids (such as malate), chelating Al^{3+} to form non-toxic complexes (Ryan et al. 2001). The *ALMT1* gene, encoding a membrane protein that facilitates an Al^{3+}-activated malate efflux when expressed in tobacco, rice, barley and *Xenopus* oocytes (Delhaize et al. 2004; Sasaki et al. 2004), has been isolated recently. However, the action spectrum of Al^{3+} appears to be much broader than just activation of anion channels responsible for malate efflux. Al^{3+} has the potential to affect membrane potential significantly by directly interacting with the membrane to alter its structure and fluidity (Chen et al. 1991), by blocking Ca^{2+} and K^+ channels (Ding et al. 1993; Gassmann and Schroeder 1994) or by inhibiting the H^+-ATPase in the plasma membrane (Ahn et al. 2002). These changes in membrane potential will inevitably affect cellular ionic homeostasis.

Al^{3+} has been shown to block inward-rectifying K^+ channels (KIR) in root hairs (Gassmann and Schroeder 1994) and guard cells (Schroeder et al. 1994), reducing the open probability and changing the activation kinetics of the channel through some internal membrane-delimited mechanism (Liu and Luan 2001). Therefore, it was suggested that K^+ deficiency in acid soils can be correlated with the inhibitory effect of Al^{3+} on KIR in roots (Gassmann and Schroeder 1994). However, it has also been shown that Al^{3+} has no effect on K^+ accumulation at the whole-plant level (Marschner 1995). Alternatively, Zsoldos et al. (2001) reported that Al^{3+} stimulated K^+ (^{86}Rb) influx in short-term (up to 6 h) experiments. This controversy might be resolved if the effect of Al^{3+} on channel gating and activation kinetics is rather different in situ than when expressed in heterologous conditions (Liu and Luan 2001). It is worth mentioning that the pH-sensitivity of SKT1 (K^+ inward-rectifying channel from potato) was rather different when expressed in insect cells versus *Xenopus* oocytes, indicating that the heterologous expression system influences the functional behavior of the channel (Hartje et al. 2000). From this point of view, non-invasive ion flux measurements are ideal to study effects of Al^{3+} on ion transport across membranes.

Several studies have now demonstrated that the Al^{3+}-activated malate efflux is accompanied by K^+ efflux and it has been proposed that this can account for electroneutrality (Ryan et al. 1995). However, all these studies used excised root apices and relatively short treatment times and it remains unclear whether a similar response operates in intact roots and if it is sustained over long periods of Al^{3+} exposure. In our recent work we used near-isogenic wheat lines that differ in Al^{3+} tolerance (ET8 and ES8) to investigate the effects of Al^{3+} on root-cell membrane potential and ion flux kinetics in intact roots (Wherrett et al. 2005). We showed that addition of 50 μM $AlCl_3$ to the bathing solution stimulated an increase in K^+ efflux and H^+ influx in ET8 but not in ES8. The differences between the genotypes were sustained for

24 h and were observed only at the elongating zone and not the meristematic zone (Wherrett et al. 2005). These results provide new temporal and spatial information on the Al^{3+}-activated efflux of K^+ from intact wheat plants. Based on our MIFE data, we also concluded that a previously proposed model (Olivetti et al. 1995) to explain the Al^{3+}-dependent depolarization in snapbean (blockage of K^+ outward rectifying (KOR) channel by Al^{3+}) is unlikely to be occurring in wheat because the depolarization in ET8 are associated with a transient increase in K^+ efflux, not a decrease as would be predicted from that model. Instead, our data suggested no direct interaction between Al^{3+} and K^+ channels and implied that the stimulation of K^+ efflux is triggered by the membrane depolarization caused by organic anion efflux. Aluminum also caused significant changes in the net H^+ and Ca^{2+} fluxes (Wherrett et al. 2005). The physiological role of these changes is the subject of further investigation.

As mentioned above, no Al^{3+} effect on K^+ accumulation was observed at the whole-plant level (Marschner 1995). That points to the possibility that the soil (or apoplastic) pH per se may influence K^+ uptake. It is known that Shaker-type K^+ channels are controlled (at post-transcriptional level) by both pH_i and pH_0 (Marten et al. 1999; Lacombe et al. 2000).

The most studied channels are K^+ channels in guard cells. Both apoplasmic (Roelfsema and Hedrich 2002) and cytosolic (Dietrich et al. 2001) acidification lead to the activation of inward K^+ currents in guard cells. The effect is voltage-dependent (Roelfsema and Hedrich 2002). Strong pH-dependence of KIR channels was also reported for root (tomato LKT1 channel; Hartje et al. 2000), pulvini (Yu et al. 2001) and mesophyll (Keunecke and Hansen 2000) cells. Results are rather controversial. While LCT1 channel behavior was similar to KIR in guard cells, the pulvinar KIR channels were inhibited by external acidification, with reversible decline of the maximum conductance and an irreversible shift of the voltage dependence of channel gating (Yu et al. 2001), in contrast to their acidification-promoted counterparts in guard cells. In leaf tissues, acidification stimulated K^+ conductance in bundle sheath cells, whereas a decrease was found for mesophyll channels (Keunecke and Hansen 2000). Also, extracellular acidification decreased the macroscopic currents through the AKT3 channel (weakly voltage-dependent channel predominantly expressed in the phloem) by reducing the single-channel conductance (Marten et al. 1999).

Equally controversial are data on pH regulation of KOR channels. Reports range from strong inhibition (Ilan et al. 1994; Lacombe et al. 2000) to insensitivity (Fan et al. 2003). There is also no agreement on the above mechanisms of pH control of K^+ channels. Hoth et al. (2001) showed that essential elements for external pH regulation are located within the channel pore. At the same time, no evidence for pH effects on single channel conductance was reported (Ilan et al. 1994; Lacombe et al. 2000).

The fact that external protons can show opposite effects on different members of the same gene family is hardly surprising as most of the results originate from experiments with heterologously expressed K^+ channels. Amtmann et al. (1999) provided the first *in planta* characterization of the effects of

apoplastic pH on KIR channels in roots, showing that a decrease in external pH shifted the half-activation potential to more positive voltages and increased the limit conductance, resulting in enhancement of the KIR current. Increased probability of the KIR opening was also shown in patch-clamp experiments by Hartje et al. (2000). This is opposite to what would be expected if K^+ deficiency in roots grown at low pH is due to direct regulation of KIR channels by protons. More likely, K^+ acquisition is under the strict control of the plasma membrane potential which, in turn, is pH-sensitive. Indeed, acidifying bathing media to pH 4 leads to significant H^+ influx into the plant tissue (Shabala et al. 1997), which can potentially explain cytoplasmic acidification observed in *Riccia* rhizoids (Felle 1988) and in protoplasts from wheat roots (Lindberg and Strid 1997). At the same time, most K^+ channels in the plant plasma membrane (with the only exception of AKT2/3) show strong potential dependence (Very and Sentenac 2002; Shabala 2003).

The above hypothesis that K^+ deficiency at low pH may result from direct control over K^+ transport by the depolarized MP was confirmed in experiments, when net K^+ and H^+ fluxes near *Arabidopsis* root hairs were measured under voltage clamped conditions and in a range of different external pH and KCl concentrations (Babourina et al. 2001), providing strong evidence that K^+ acquisition by root hairs is under strict voltage control and supporting the above hypothesis on mediation by membrane potential of soil pH effects on K^+ acquisition.

3.4.6 Oxygen deprivation

Many agricultural regions around the world are subject to waterlogging, caused either by natural factors or as a result of excessive irrigation (Gibbs and Greenway 2003). In waterlogged soils, air is displaced from the pore spaces, and oxygen is rapidly depleted, which changes the root chemical environment and affects root growth and causes a decline in acquisition of major nutrients such as N, P, K and Ca (Atwell and Steer 1990; Boem et al. 1996; Barrett-Lennard et al. 1999). Low oxygen levels in the rhizosphere and an associated significant (up to 97%; Gibbs and Greenway 2003) reduction in the rate of energy production are believed to be the main reason for that decline. In addition, long-term waterlogging reduces soil redox potential, causing dramatic increase in the levels of free Fe^{2+} and Mn^{2+}, leading to toxicity of these nutrients for root metabolism (Marschner 1995).

It has frequently been suggested that anoxia decreases membrane selectivity. For example, Armstrong and Drew (2002) showed that low concentration of oxygen in the rooting medium decreases the selectivity of K^+/Na^+ uptake in favor of Na^+ and retards the transport of K^+ to the shoots. This may lead to a several fold increase in Na^+ content in leaf tissues compared with normoxic plants (Smethurst and Shabala 2003). A so called "memory effect", when enhanced shoot transport of Na^+ remains present for many days even after

short-term (1 h) oxygen deficiency, is known from the literature (Brauer et al. 1987). The precise ionic mechanisms involved remain to be elucidated. It also remains unknown whether this decreased selectivity is due to an increased permeability of the lipid bilayer or is caused by leakage of ions through some specific channels or carriers (Greenway and Gibbs 2003).

Despite maintenance of membrane integrity being considered as a key factor in survival of plant cells under anoxia (Rawyler et al. 1999), surprisingly little is known about processes that maintain membrane integrity under anoxic conditions. Non-specific loss of K^+ was reported soon after onset of anoxia (Greenway et al. 1992; Colmer et al. 2001), presumably as a result of plasma membrane depolarization leading to KOR opening (Zhang et al. 1992; Zhang and Greenway 1995). It was speculated that cells direct their limited amounts of energy to the transport of solutes involved in pH regulation and of sugars required for anaerobic carbohydrate catabolism (Greenway and Gibbs 2003). As a result, net uptake of other nutrients (K^+, PO_4^{-3}, Cl^-, and NH_4^+) is strongly inhibited (Petraglia and Poole 1980; Colmer et al. 2001).

Elucidating the signaling cascade of plant responses to anoxia remains one of the great challenges. It was suggested that cytosolic acidification is the primary signal of an O_2 deficit (Ratcliffe 1997; Gibbs and Greenway 2003). Xia and Roberts (1996) provided the evidence that plasma membrane H^+-ATPase can operate under anoxia and that net H^+ extrusion increases when cytosolic pH falls. However, Felle (1996) concluded that changes in pump activity do not affect cytoplasmic pH under anoxic conditions. Changes in $[Ca^{2+}]_{cyt}$ are also thought to be part of the signal transduction pathway (Subbaiah et al. 1998).

Greenway and Gibbs (2003) proposed that in anoxia-tolerant tissues, energy flow during anoxia must be directed towards essential nutrient transport. No direct evidence has been provided though. From this point of view, non-invasive ion flux measurements give an excellent opportunity to look at underlying mechanisms associated with membrane responses to O_2 deprivation. Results of recent studies in our laboratory have revealed that functionally different barley root zones have rather different O_2 requirements (as measured with vibrating O_2 microelectrode) and that oxygen deprivation has qualitatively different effects on the activity of plasma membrane ion transporters in mature and elongation zones. Based on pharmacological data, we suggest that hypoxia-induced K^+ flux responses are mediated by both KIR and NSCC channels in the elongation zone, while in the mature zone KOR channels are likely to be the key (J. Pang and S. Shabala, in preparation). The ion flux "signatures" in response to hypoxia were rather different between cultivars contrasting in waterlogging tolerance, suggesting that the latter was mediated not only by anatomical (e.g. aerenchyma formation) but also physiological mechanisms.

3.4.7 Oxidative stress

Reactive oxygen species (ROS) are produced as a by-product of cellular metabolic pathways and function as a critical second messenger in a variety of

intracellular signaling pathways in animal tissues (Kourie 1998). Plants also respond to both biotic (elicitors) and abiotic (salinity, drought, chilling) stresses by the production of reactive oxygen species such as the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\bullet}$) (Bowler and Fluhr 2000; Smirnoff and Wheeler 2000). It is traditionally believed that increased ROS production has detrimental effects on cell ionic homeostasis, causing lipid peroxidation and impairing membrane integrity (Santos et al. 2001; Lee et al. 2004). However, there is growing evidence that, in addition to having detrimental effects, ROS can also be important signaling and regulatory agents. Examples are numerous and include ROS involvement in plant signal transduction (Pei et al. 2000; Schroeder et al. 2001; Zhang et al. 2001), development (Shorning et al. 2000), and programmed cell death (Hoeberichts and Woltering 2003; Casolo et al. 2005).

Most evidence on the signaling role of ROS comes from experiments on guard cells. ROS have been proposed to function as second messengers in ABA signaling in guard cells (Kwak et al. 2003; Jiang and Zhang 2003). It is believed that in such a signaling cascade, ROS are produced by NAD(P)H oxidases (Jiang and Zhang 2003; Kwak et al. 2003), and this process is preceded by cytoplasmic alkalization (Suhita et al. 2004). The intracellular signaling pathway is rather complex and involves activation of a heterotrimeric G protein, H_2O_2 generation, and changes in $[Ca^{2+}]_{cyt}$ in the regulation of stomatal movements (Chen et al. 2004). However, Kohler et al. (2003) showed that physiological concentrations of H_2O_2 strongly inhibited K^+ outward rectifiers in *Vicia* guard cells. These findings question the role of H_2O_2 as a critical second messenger regulating guard cell ion channels in response to ABA. Obviously, the signaling network is more complex than initially believed, and may include a variety of different types of membrane transporters and second messengers. To understand their orchestrated functions remains a great challenge.

By using the non-invasive MIFE technique we showed, for the first time, that free hydroxyl radicals ($OH^{\bullet}$) caused rapid Ca^{2+} influx and K^+ efflux across the plasma membrane of *Arabidopsis* root cells (Demidchik et al. 2003). Patch-clamp and luminometry experiments suggested that this effect is mediated by ROS control upon the activity of at least two different types of plasma membrane channels. ROS-induced increase in cytosolic Ca^{2+} was mediated by a novel population of NSCC that differ in selectivity and pharmacology from those involved in toxic Na^+ influx (Demidchik and Tester 2002), while ROS-induced K^+ efflux was due to $OH^{\bullet}$ stimulation of a KOR. This is the first characterization of the channel-mediated K^+ efflux mechanism of plant stress responses and is in contrast to H_2O_2 data. Experiments with *abi1* mutant suggested that the phosphorylation state is critical to such KOR activation (Demidchik et al. 2003).

Interaction between ROS and $[Ca^{2+}]_{cyt}$ appears to be critical in mediating plant stress responses (Bowler and Fluhr 2000; Coelho et al. 2002). Modulation of Ca^{2+} permeable channels by H_2O_2 in guard cells, leading to stomatal closure, has been reported widely (Pei et al. 2000; Murata et al. 2001;

Schroeder et al. 2001; Zhang et al. 2001; Kwak et al. 2003). It was shown that this process requires the presence of NAD(P)H in the cytosol (Murata et al. 2001). However, little is known about the subcellular spatio–temporal patterns of ROS production or their significance for downstream responses (Coelho et al. 2002).

Effects of ROS on K^+ channels are well documented in both animal and plant literature. Most studies deal with either mitochondrial K^+-ATP channels (Trono et al. 2004; Casolo et al. 2005) or with K^+ permeable channels in guard cell plasma membranes (Zhang et al. 2001; Kohler et al. 2003). Both of these were suggested to play a role as defense systems under environmental stress. Mitochondrial K^+-ATP channels were activated under stress conditions (salinity and osmotic stress) and were able to control mitochondrial superoxide anion production (Trono et al. 2004).

Potassium efflux is known to be one of the earliest events observed in response to a variety of stresses such as salinity (Shabala 2000; Babourina et al. 2001; Shabala et al. 2003) and acidity (Babourina et al. 2001). Traditionally, these effects were attributed to membrane depolarization (Shabala et al. 2003). Our study of K^+ efflux from plant roots in response to oxidative stress (Demidchik et al. 2003) demonstrated that stress-induced K^+ efflux can be mediated by a previously unknown mechanism—activation of KOR by $OH^\bullet$. In fact, this is hardly surprising. K^+ channels harbor reactive groups and thus are expected to be sensitive to ROS (Kohler et al. 2003). Importantly, flux amplitude and the time-course of K^+ flux response to ROS treatment varied between species, suggesting species-specific "flux signatures" in response to $OH^\bullet$. This also suggests that H_2O_2 is not the sole oxygen-derived species capable of signaling and regulation in plants. The response to $OH^\bullet$ was tissue-specific and stronger in cells which directly interact with the environment (e.g. root epidermis versus pericycle). Based on the above results, two major functions for ROS activation of cation channels were proposed: initialization/amplification of stress signals and control of cell elongation in root growth (Demidchik et al. 2003).

3.4.8 Biotic stresses

Plants respond to attack from pathogens by activating a variety of defense mechanisms, including synthesis of phytoalexins and hypersensitive cell death, which restricts growth of pathogens at the site of infection (Kadota et al. 2004). These responses are preceded by the interaction between pathogen-associated molecules (elicitors) and putative plant receptors (Vera-Estrella et al. 1994; Blumwald et al. 1998). Some of the earliest detectable signaling events in plant defense responses include plasma membrane depolarization and transmembrane ion fluxes, followed by production of ROS (Zimmermann et al. 1998; Clough et al. 2000). These are sequentially followed by defense gene activation and phytoalexin accumulation (Jabs et al. 1997). Most papers

suggest elicitor-induced Ca^{2+} and H^+ influx and effluxes of Cl^- and K^+ (Nurnberger et al. 1994; Jabs et al. 1997; Kadota et al. 2004).

Of particular importance in early recognition between the host and pathogen is the role of Ca^{2+} as a second messenger that triggers a downstream cascade of defense responses (Blumwald et al. 1998; Zimmermann et al. 1999). Fungal elicitors rapidly enhanced expression of the plasma membrane Ca^{2+} pump in soybean (Chung et al. 2000). Ca^{2+} influx and the transient increase in $[Ca^{2+}]_{cyt}$ levels after elicitor treatment have been shown to be necessary and sufficient for the induction of an oxidative burst and thus, plant defense responses (Clough et al. 2000). The important role of Ca^{2+} signaling in response to pathogen infection was observed in a wide range of species (Bach et al. 1993; Nurnberger et al. 1994; Jabs et al. 1997; Blume et al. 2000; Lecourieux et al. 2002; Kadota et al. 2004). Surprisingly, despite the great bulk of literature reporting the critical role of Ca^{2+} in the early recognition between the host and pathogen, direct measurements of Ca^{2+} flux into a single infected cell in vivo are lacking. This is largely due to the lack of appropriate techniques used (as explained below).

Most of above conclusions were derived from either $^{45}Ca^{2+}$ uptake experiments (Bach et al. 1993; Nurnberger et al. 1994) or from experiments with various Ca^{2+} channel blockers (Atkinson et al. 1990; Nurnberger et al. 1997). The above pharmacological data do not provide direct evidence that specific plasma membrane Ca^{2+} channels mediate an elicitor-induced Ca^{2+} influx, because the high (millimolar) concentrations of La^{3+} or Gd^{3+} used to block Ca^{2+} influx may enter the cell and affect the plasma membrane Ca^{2+}-ATPase (Blume et al. 2000). As for the $^{45}Ca^{2+}$ uptake technique, it has a relatively low time resolution and is only capable of measuring cell-associated (not localized) Ca^{2+} uptake (Grant et al. 2000), precluding accurate quantification of Ca^{2+} influx into the cell and adequate resolution of the fast kinetics associated with early signaling events at the plasma membrane.

Another popular technique is the use of aequorin technology, with a large number of papers published on kinetics of $[Ca^{2+}]_{cyt}$ changes in many plant species in response to various elicitors and phytotoxins (Blume et al. 2000; Grant et al. 2000; Lecourieux et al. 2002). The main problem with this method is, however, that it cannot distinguish between transient $[Ca^{2+}]_{cyt}$ changes occurring as a result of increased Ca^{2+} uptake from the external medium (i.e. plasma membrane mediated) and changes resulting from Ca^{2+} release from internal store (such as vacuole or ER). Thus, the use of aequorin technology does not allow location of the specific elicitor target(s) in plant cells.

Direct electrophysiological evidence of elicitor-induced activation of plasma membrane Ca^{2+} permeable channels is scarce (Gelli et al. 1997; Zimmermann et al. 1997) and is usually obtained from patch-clamp experiments. However, as protoplasts are highly artificial systems, it is unclear to what extent these results are applicable to cellular responses in vivo (e.g. Mathieu et al. 1991 versus Zimmerman et al. 1999). Several explanations are possible including (i) indirect regulation of channel activity by an elicitor,

(ii) the loss of responsiveness of the enzymatically isolated protoplasts, and (iii) the end target of toxin being in the cell wall. In each of these cases, the use of the MIFE technique will not be prone to these problems.

In a recent study, we applied the MIFE technique to characterize early signaling events associated with thaxtomin A (a dipeptide phytotoxin produced by all plant pathogenic *Streptomyces* sp. responsible for common scab disease) toxicity in *Arabidopsis* and tomato roots and pollen tubes. Our results indicate that thaxtomin A treatment causes Ca^{2+}-channel-mediated rapid Ca^{2+} influx across the plasma membrane, triggering further Ca^{2+}-induced Ca^{2+} release from some internal store (Tegg et al. 2005). We also showed that thaxtomin A was more effective in young, physiologically active tissues, suggesting higher density of toxin-binding sites in these regions, as well as suggesting a possible interaction between thaxtomin A and plasma membrane auxin receptors, as revealed from experiments on the auxin sensitive *ucu2–2/gi2 Arabidopsis* mutant (Tegg et al. 2005).

Not only Ca^{2+} but fluxes of other ions such as H^+, K^+ and Cl^- are also affected by pathogens (Zimmermann et al. 1999; Clough et al. 2000; Lecourieux et al. 2002; Kadota et al. 2004) and have been implicated in early pathogen recognition. In contrast to Ca^{2+}, effects of elicitors on H^+ flux activity across the plasma membrane are more variable and often controversial. Generally, extracellular alkalinization occurs when plant cells are treated with an elicitor (Atkinson et al. 1990; Nurnberger et al. 1994). However, some papers report significant (up to 4-fold) activation of the plasma membrane H^+-ATPase (Vera-Estrella et al. 1994; Blumwald et al. 1998). This controversy may be explained by the lack of direct measurements of elicitor-induced H^+ fluxes, as most conclusions were derived from observed pH changes in the cell medium.

It remains to be answered what specific membrane proteins mediate elicitor perception and signaling in plants. Various K^+ permeable inward- and outward rectifying channels have been named as potential targets (Blatt et al. 1999; Ivashikina et al. 2001). A series of recent publications using *dnd* (Clough et al. 2000) and *hlm* (Balague et al. 2003) *Arabidopsis* mutants pointed to the involvement of the cyclic nucleotide-gated non-selective ion channels (CNG). Such CNG channels are known to be permeable to all physiologically relevant cations and thus, might mediate the observed elicitor-induced Ca^{2+} and K^+ flux changes. This hypothesis can be easily tested in MIFE experiments on these mutants.

In addition to plant-pathogenic interactions, early electrophysiological events at the plasma membrane are also critical for root nodulation (Felle et al. 1995; Ehrhardt et al. 1996; Zimmermann et al. 1999). Membrane potential changes and ion fluxes were both shown to be of importance for Nod signal perception and transduction (Zimmermann et al. 1999). Specific details of this process remain to be elucidated. It was shown that adding nodulation signaling protein NodO to a planar lipid bilayer causes formation of cation-selective channels that allow K^+ and Na^+ fluxes across the membrane (Sutton

et al. 1994). The full details of this process, as well as the overall role of ion channels during the establishment of symbiosis, remain to be answered (Zimmermann et al. 1999). Non-invasive ion flux measurements might be an excellent tool for doing this.

3.5 Prospects and conclusions

As shown above, key features of the MIFE technique (such as non-invasiveness, high spatial and temporal resolution, and possibility of concurrent measurements of fluxes of several ions) make it a very useful tool to study membrane-transport processes in response to literally every known environmental stress. As ion fluxes are measured in situ, a direct extrapolation of the results to a variety of "natural" situations (e.g. plant stress response in the field) becomes possible.

The power of the MIFE technique is enhanced many fold when used in combination with other advanced experimental tools. One such example is a combination of the MIFE and patch-clamp techniques (Tyerman et al. 2001; Demidchik et al. 2003). Such a combination facilitates better identification of membrane currents and makes it possible to determine stoichiometries of transporter reactions. Another example is a combination of MIFE and fluorescence imaging techniques. Shabala et al. (2002) used such an approach to monitor H^+ flux kinetics across the plasma membrane of *Listeria* bacterial cells in relation to observed intracellular pH_i changes. These authors showed that pH_i and H^+ flux responses were complimentary and reflected the key role of active H^+ extrusion in bacterial adaptation to acid stress. There is no doubt that similar measurements can also be performed on plant tissues, using a variety of dyes to monitor kinetics of the same ion on both side of the plasma membrane. Alternatively, impaled ion-selective microelectrodes may be used in combination with MIFE for these purposes. Earlier we used a similar approach to measure net ion fluxes concurrently with membrane potential changes from leaf epidermal and mesophyll cells in response to light (Shabala and Newman 1999). Combination of the MIFE technique with voltage-clamp (Babourina et al. 2001; Shabala and Lew 2002) or pressure-probe (Shabala and Lew 2002) techniques also gave a significant conceptual advance in our knowledge of the ion-transport processes underlying plant adaptive responses to pH and osmotic stresses.

Most importantly, the MIFE technique is ideal for functional genomics studies. Its application to *Arabidopsis phot* mutants led to the discovery of an important role of plasma membrane Ca^{2+} fluxes in mediating blue light induced phototropic responses (Babourina et al. 2002). Altered ion flux patterns were also reported for newly described *mtr-1* pea mutant with modified tropic responses (Platten et al. 2004) or from *Arabidopsis sos* mutants in response to salt stress (Shabala et al. 2005c). Several more projects are currently at various

stages of completion in our lab, using genetically modified material. Results appear to be very promising.

To summarize, I believe that non-invasive ion flux measurements have a unique role in filling the gap between fundamental membrane-transport studies at the molecular levels and the needs of agronomists, aiming to improve plant performance in the field under stress conditions. Although not perfect, such a technique can probably claim the "best value for money" title in its area. Time will judge if this claim is fully justified.

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References

Abbott ML, Fraley L (1991) Radiotracer methods to determine root distribution—a review. Environ Exp Bot 31:1–10

Ahn SJ, Sivaguru M, Chung GC, Rengel Z, Matsumoto H (2002) Aluminium-induced growth inhibition is associated with impaired efflux and influx of H^+ across the plasma membrane in root apices of squash (*Cucurbita pepo*). J Exp Bot 53:1959–1966

Amtmann A, Jelitto TC, Sanders D (1999) K^+-selective inward-rectifying channels and apoplastic pH in barley roots. Plant Physiol 120:331–338

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science 285:1256–1258

Armstrong W, Drew MC (2002) Root growth and metabolism under oxygen deficiency. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. Dekker, New York, pp 729–761

Atkinson MM, Keppler LD, Orlandi EW, Baker CJ, Mischke CF (1990) Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco. Plant Physiol 92:215–221

Atwell BJ, Steer BT (1990) The effect of oxygen deficiency on uptake and distribution of nutrients in maize plants. Plant Soil 122:1–8

Ayala F, Oleary JW, Schumaker KS (1996) Increased vacuolar and plasma membrane H^+-ATPase activities in *Salicornia bigelovii* Torr in response to NaCl. J Exp Bot 47:25–32

Babourina O, Leonova T, Shabala S, Newman I (2000) Effect of sudden salt stress on ion fluxes in intact wheat suspension cells. Ann Bot 85:759–767

Babourina O, Hawkins B, Lew RR, Newman I, Shabala S (2001) K^+ transport by *Arabidopsis* root hairs at low pH. Austral J Plant Physiol 28:635–641

Babourina O, Newman I, Shabala S (2002) Blue light-induced kinetics of H^+ and Ca^{2+} fluxes in etiolated wild-type and phototropin-mutant *Arabidopsis* seedlings. Proc Natl Acad Sci USA 99:2433–2438

Bach M, Schnitzler J-P, Seitz HU (1993) Elicitor-induced changes in Ca^{2+} influx, K^+ efflux, and 4-hydroxybenzoic acid synthesis in protoplasts of *Daucus carota* L. Plant Physiol 103:407–412

Bajaj S, Targolli J, Liu LF, Ho THD, Wu R (1999) Transgenic approaches to increase dehydration-stress tolerance in plants. Mol Breeding 5:493–503

Balague C, Lin B, Alcon C, Flottes G, Malmstrom S, Kohler C, Neuhaus G, Pelletier G, Gaymard F, Roby D (2003) HLM1, an essential signalling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell 15:365–379

Barrett-Lennard EG, Ratingen PV, Mathie MH (1999) The developing pattern of damage in wheat (*Triticum aestivum* L.) due to the combined stresses of salinity and hypoxia:

experiments under controlled conditions suggest a methodology for plant selection. Aust J Agricult Res 50:129–136
Bisson MA, Gutknecht J (1977) Osmotic regulation in the marine alga, *Codium decorticatum*. II. Active chloride influx exerts negative feedback control on the turgor pressure. J Membrane Biol 37:85–98
Blatt MR, Grabov A, Brearley J, Hammond-Kosack K, Jones JDG (1999) K^+ channels of Cf-9 transgenic tobacco guard cells as targets for *Cladosporium fulvum* Avr9 elicitor-dependent signal transduction. Plant J 19:453–462
Blume B, Nürnberger T, Nass N, Scheel D (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 12:1425–1440
Blumwald E, Aharon GS, Lam C-H (1998) Early signal transduction pathways in plant-pathogen interactions. Trends Plant Sci 3:342–346
Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. Biochim Biophys Acta 1465:140–151
Boem FHG, Lavado RS, Porcelli CA (1996) Note on the effects of winter and spring waterlogging on growth, chemical composition and yield of rapeseed. Field Crop Res 47:175–179
Bohnert HJ, Shen B (1999) Transformation and compatible solutes. Sci Hort 78:237–260
Bohnert HJ, Sheveleva E (1998) Plant stress adaptations—making metabolism move. Curr Opin Plant Biol 1:267–274
Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptation to environmental stresses. Plant Cell 7:1099–1111
Bowler C, Fluhr R (2000) The role of calcium and activated oxygen as signals for controlling cross-tolerance. Trend Plant Sci 5:241–246
Brauer D, Leggett JE, Egli DB (1987) Changes in K, Rb and Na transport to shoots after anoxia. Plant Physiol 83:219–224
Bravo-F P, Uribe EG (1981) An analysis of the relationship between respiration and the transmembrane potential in corn roots. Plant Physiol 67:809–814
Brownlee C, Goddard H, Hetherington AM, Peake L-A (1999) Specificity and integration of responses: Ca^{2+} as a signal in polarity and osmotic regulation. J Exp Bot 50:1001–1011
Carden DE, Walker DJ, Flowers TJ, Miller AJ (2003) Single-cell measurements of the contributions of cytosolic Na^+ and K^+ to salt tolerance. Plant Physiol 131:676–683
Casolo V, Petrussa E, Krajnakova J, Macri F, Vianello A (2005) Involvement of the mitochondrial K^+-ATP channel in H_2O_2- or NO-induced programmed death of soybean suspension cell cultures. J Exp Bot 56:997–1006
Chen J, Sucoff EI, Stadelmann EJ (1991) Aluminum and temperature alteration of cell membrane permeability of *Quercus rubra*. Plant Physiol 96:644–649
Chen YL, Huang RF, Xiao YM, Lu P, Chen J, Wang XC (2004) Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and H_2O_2. Plant Physiol 136:4096–4103
Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K^+ flux: a case study for barley. Plant Cell Environ 28:1230–1246
Chung WS, Lee SH, Kim JC, Heo WD, Kim MC, Park CY, Park HC, Lim CO, Kim WB, Harper JF, Cho MJ (2000) Identification of a calmodulin-regulated soybean Ca^{2+}-ATPase (SCA1) that is located in the plasma membrane. Plant Cell 12:1393–1407
Clarkson DT, Earnshow MJ, White PJ, Cooper HD (1988) Temperature dependent factors influencing nutrient uptake: an analysis of responses at different levels of organization. Plants and temperature. Society for Experimental Botany, Cambridge, pp 281–309
Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK, Bent AF (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proc Natl Acad Sci USA 97:9323–9328
Coelho SM, Taylor AR, Ryan KP, Sousa-Pinto I, Brown MT, Brownlee C (2002) Spatiotemporal patterning of reactive oxygen production and Ca^{2+} wave propagation in fucus rhizoid cells. Plant Cell 14:2369–2381

Colmer TD, Bloom AJ (1998) A comparison of NH_4^+ and NO_3^- net fluxes along roots of rice and maize. Plant Cell Environ 21:240–246

Colmer TD, Huang SB, Greenway H (2001) Evidence for down-regulation of ethanolic fermentation and K^+ effluxes in the coleoptile of rice seedlings during prolonged anoxia. J Exp Bot 52:1507–1517

Cramer GR, Lynch J, Lauchli A, Epstein E (1987) Influx of Na^+, K^+, and Ca^{2+} into roots of salt-stressed cotton seedlings. Effects of supplemental Ca^{2+}. Plant Physiol 83:510–516

Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H (2004) Engineering high-level aluminum tolerance in barley with the ALMT1 gene. Proc Natl Acad Sci USA 101:15249–15254

Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from *Arabidopsis* roots. Plant Physiol 128:379–387

Demidchik V, Shabala SN, Coutts KB, Tester MA, Davies JM (2003) Free oxygen radicals regulate plasma membrane Ca^{2+} and K^+-permeable channels in plant root cells. J Cell Sci 116:81–88

Dietrich P, Sanders D, Hedrich R (2001) The role of ion channels in light-dependent stomatal opening. J Exp Bot 52:1959–1967

Ding JP, Badot PM, Pickard BG (1993) Aluminium and hydrogen ions inhibit a mechanosensory calcium-selective cation channel. Aust J Plant Physiol 20:771–778

DuPont FM (1989) Effect of temperature on the plasma membrane and tonoplast ATPases of barley roots. Plant Physiol 89:1401–1412

Ehrhardt DW, Wais R, Long SR (1996) Calcium spiking in plant root hairs responding to rhizobium nodulation signals. Cell 85:673–681

Elphick CH, Sanders D, Maathuis FJM (2001) Critical role of divalent cations and Na^+ efflux in *Arabidopsis thaliana* salt tolerance. Plant Cell Environ 24:733–740

Fan LM, Wang YF, Wu WH (2003) Outward K^+ channels in *Brassica chinensis* pollen protoplasts are regulated by external and internal pH. Protoplasma 220:143–152

Felle HH (1988) Short-term pH regulation in plants. Physiol Plant 74:583–591

Felle HH (1996) Control of cytoplasmic pH under anoxic conditions and its implication for plasma membrane proton transport in *Medicago sativa* root hairs. J Exp Bot 47:967–973

Felle HH, Kondorosi E, Kondorosi A, Schultze M (1995) Nod signal-induced plasma-membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. Plant J 7:939–947

Garnett TP, Shabala SN, Smethurst PJ, Newman IA (2001) Simultaneous measurement of ammonium, nitrate and proton fluxes along the length of eucalypt roots. Plant Soil 236:55–62

Garnett TP, Shabala SN, Smethurst PJ, Newman IA (2003) Kinetics of ammonium and nitrate uptake by eucalypt roots and associated proton fluxes measured using ion selective microelectrodes. Funct Plant Biol 30:1165–1176

Garril A, Davies JM (1994) Patch clamping fungal membranes: new perspectives on ion transport. Micol Res 98:257–263

Gassmann W, Schroeder JI (1994) Inward-rectifying K^+ channels in root hairs of wheat—a mechanism for aluminum-sensitive low-affinity K^+ uptake and membrane potential control. Plant Physiol 105:1399–1408

Gelli A, Higgins VJ, Blumwald E (1997) Activation of plant plasma membrane Ca^{2+}-permeable channels by race-specific fungal elicitors. Plant Physiol 113:269–279

Gibbs J, Greenway H (2003) Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. Funct Plant Biol 30:1–47

Gilroy S, Jones DL (2000) Through form to function: root hair development and nutrient uptake. Trend Plant Sci 5:56–60

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J 23:441–450

Greenway H, Gibbs J (2003) Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. Funct Plant Biol 30:999–1036

Greenway H, Waters I, Newsome J (1992) Effects of anoxia on uptake and loss of solutes in roots of wheat. Aust J Plant Physiol 19:233–247

Hällgren J-E, Öquist G (1990) Adaptation to low temperatures. In: Alscher RG, Cumming JR (eds) Stress responses in plants: adaptation and acclimation mechanisms. Wiley-Liss, New York, pp 265–293

Handson PD, Shelley BC (1993) A review of plant analysis in Australia. Aust J Exp Agricult 33:1029–1038

Hartje S, Zimmermann S, Klonus D, Mueller-Roeber B (2000) Functional characterisation of LKT1, a K^+ uptake channel from tomato root hairs, and comparison with the closely related potato inwardly rectifying K^+ channel SKT1 after expression in *Xenopus oocytes*. Planta 210:723–731

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499

Henriksen GH, Bloom AJ, Spanswick RM (1990) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. Plant Physiol 93:271–280

Henriksen GH, Raman DR, Walker LP, Spanswick RM (1992) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. 2. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. Plant Physiol 99:734–747

Hoeberichts FA, Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays 25:47–57

Hoth S, Geiger D, Becker D, Hedrich R (2001) The pore of plant K^+ channels is involved in voltage and pH sensing: domain-swapping between different K^+ channel alpha-subunits. Plant Cell 13:943–952

Ilan N, Schwartz A, Moran N (1994) External pH effects on the depolarization-activated K^+-channels in guard-cell protoplasts of *Vicia faba*. J Gen Physiol 103:807–831

Ivashikina N, Becker D, Ache P, Meyerhoff O, Felle HH, Hedrich R (2001) K^+ channel profile and electrical properties of *Arabidopsis* root hairs. FEBS Lett 508:463–469

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci USA 94:4800–4805

Jiang M, Zhang J (2003) Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defence in leaves of maize seedlings. Plant Cell Environ 26:929–939

Jones DL, Shaff JE, Kochian LV (1995) Role of calcium and other ions in directing root hair tip growth in *Limnobium stoloniferum*. 1. Inhibition of tip growth by aluminum. Planta 197:672–680

Kadota Y, Goh T, Tomatsu H, Tamauchi R, Higashi K, Muto S, Kuchitsu K (2004) Cryptogein-induced initial events in tobacco BY-2 cells: Pharmacological characterization of molecular relationship among cytosolic Ca^{2+} transients, anion efflux and production of reactive oxygen species. Plant Cell Physiol 45:160–170

Keunecke M, Hansen UP (2000) Different pH-dependences of K^+ channel activity in bundle sheath and mesophyll cells of maize leaves. Planta 210:792–800

Kinraide TB, Wyse RE (1986) Electrical evidence for turgor inhibition of proton extrusion in sugar beet taproot. Plant Physiol 82:1148–1150

Knight H, Knight MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trend Plant Sci 6:262–267

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signalling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 8:489–503

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol 46:237–260

Kohler B, Hills A, Blatt MR (2003) Control of guard cell ion channels by hydrogen peroxide and abscisic acid indicates their action through alternate signalling pathways. Plant Physiol 131:385–388

Kourie JI (1998) Interaction of reactive oxygen species with ion transport mechanisms. Am J Physiol 44:1–24

Kourie JI, Findlay GP (1991) Ionic currents across the plasmalemma of *Chara inflata* cells. III. Water-relations parameters and their correlation with membrane electrical properties. J Exp Bot 42:151–158

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signalling in *Arabidopsis*. EMBO J 22:2623–2633

Lacombe B, Pilot G, Gaymard F, Sentenac H, Thibaud JB (2000) pH control of the plant outwardly-rectifying potassium channel SKOR. FEBS Lett 466:351–354

Lecourieux D, Mazars C, Pauly N, Ranjeva R, Pugin A (2002) Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. Plant Cell 14:2627–2641

Lee SH, Singh AP, Chung GC (2004) Rapid accumulation of hydrogen peroxide in cucumber roots due to exposure to low temperature appears to mediate decreases in water transport. J Exp Bot 55:1733–1741

Lew RR (1996) Pressure regulation of the electrical properties of growing *Arabidopsis thaliana* L root hairs. Plant Physiol 112:1089–1100

Lew RR (1998) Immediate and steady state extracellular ionic fluxes of growing *Arabidopsis thaliana* root hairs under hyperosmotic and hypoosmotic conditions. Physiol Plant 104:397–404

Li Z-S, Delrot S (1987) Osmotic dependence of the transmembrane potential difference of broad bean mesocarp cells. Plant Physiol 84:895–899

Lindberg S, Strid H (1997) Aluminium induces rapid changes in the cytosolic pH and free calcium and potassium concentrations in root protoplasts of wheat (*Triticum aestivum*). Physiol Plant 99:404–414

Liu K, Luan S (1998) Voltage-dependent K^+ channels as targets of osmosensing in guard cells. Plant Cell 10:1957–1970

Liu K, Luan S (2001) Internal aluminum block of plant inward K^+ channels. Plant Cell 13:1453–1465

Lucas WJ, Kochian LV (1986) Ion transport processes in corn roots: An approach utilizing microelectrode techniques. In: Gensler WG (ed) Advanced agricultural instrumentation: design and use. Nijhoff, Dordrecht, pp 402–425

Lyons JM (1973) Chilling injury in plants. Annu Rev Plant Physiol 24:445–466

Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot 84:123–133

Macduff JH, Dhanoa MS (1996) Diurnal and ultradian rhythms in K^+ uptake by *Trifolium repens* under natural light patterns—evidence for segmentation at different root temperatures. Physiol Plant 98:298–308

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London, 889 pp

Marten I, Hoth S, Deeken R, Ache P, Ketchum KA, Hoshi T, Hedrich R (1999) AKT3, a phloem-localized K^+ channel, is blocked by protons. Proc Natl Acad Sci USA 96:7581–7586

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiol 126:1646–1667

Mathieu Y, Kurkdjian A, Xia H, Guern J, Koller A, Spiro MD, Oneill M, Albersheim P, Darvill A (1991) Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. Plant J 1:333–343

Mesnard F, Ratcliffe RG (2005) NMR analysis of plant nitrogen metabolism. Photosynth Res 83:163–180

Miller AJ, Cookson SJ, Smith SJ, Wells DM (2001) The use of microelectrodes to investigate compartmentation and the transport of metabolized inorganic ions in plants. J Exp Bot 52:541–549

Monroy AF, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell 7:321–331

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250

Murata N, Los DA (1997) Membrane fluidity and temperature perception. Plant Physiol 115:875–879

Murata Y, Pei ZM, Mori IC, Schroeder J (2001) Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1–1* and *abi2–1* protein phosphatase 2C mutants. Plant Cell 13:2513–2523

Newman IA (2001) Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. Plant Cell Environ 24:1–14

Newman IA, Kochian LV, Grusak MA, Lucas WJ (1987) Fluxes of H^+ and K^+ in corn roots. Characterisation and stoichiometries using ion-selective microelectrodes. Plant Physiol 84:1177–1184

Nurnberger T, Nennsteil D, Jabs T, Sacks WR, Hahlbrock K, Scheel D (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. Cell 78:449–460

Nurnberger T, Wirtz W, Nennstiel D, Hahlbrock K, Jabs T, Zimmermann S, Scheel D (1997) Signal perception and intracellular signal transduction in plant pathogen defence. J Recept Signal Transd Res 17:127–136

Okazaki Y, Shimmen T, Tazawa M (1984) Turgor regulation in a brackish Charophyte, *Lamprothamnium succinctum*. II. Changes in K^+, Na^+ and Cl^- concentrations, membrane potential and membrane resistance during turgor regulation. Plant Cell Physiol 25:573–581

Olivetti GP, Cumming JR, Etherton B (1995) Membrane-potential depolarization of root cap cells precedes aluminum tolerance in snapbean. Plant Physiol 109:123–129

Palmgren MG (1991) Regulation of plasma membrane H^+-ATPase activity. Physiol Plant 83:314–323

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406:731–734

Petraglia T, Poole RJ (1980) Effect of anoxia on ATP levels and ion transport rates in red beet. Plant Physiol 65:973–974

Pettersson S (1995) Low root zone temperature effects on net mineral nutrient uptake and distribution in barley (*Hordeum vulgare*). J Plant Physiol 145:459–464

Pineros MA, Shaff JE, Kochian V (1998) Development, characterization, and application of a cadmium-selective microelectrode for the measurement of cadmium fluxes in roots of *Thlaspi species* and wheat. Plant Physiol 116:1393–1401

Platten JD, Shabala SN, Elliott RC, Reid JB (2004) A novel mutant with modified tropic responses in *Pisum sativum* L. Planta 220:222–229

Pritchard J, Barlow PW, Adams JS, Tomos AD (1990) Biophysics of the inhibition of the growth of maize roots by lowered temperature. Plant Physiol 93:220–230

Ratcliffe RG (1997) In vivo NMR studies of the metabolic response of plant tissues to anoxia. Ann Bot 79:39–48

Raven JA (1985) Regulation of pH and generation of osmolarity in vascular plants: a cost-benefit analysis in relation to efficiency of use of energy, nitrogen and water. New Phytol 101:25–77

Rawyler A, Pavelic D, Gianinazzi C, Oberson J, Braendle R (1999) Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. Plant Physiol 120:293–300

Reid RJ, Smith FA (2000) The limits of sodium/calcium interactions in plant growth. Aust J Plant Physiol 27:709–715

Reid RJ, Jefferies RL, Pitman MG (1984) *Lamprothamnium*, a euryhaline charophyte. J Exp Bot 35:925–937

Reinhold L, Seiden A, Volokita M (1984) Is modulation of the rate of proton pumping a key event in osmoregulation? Plant Physiol 75:846–849

Rengel Z (1992) The role of calcium in salt toxicity. Plant Cell Environ 15:625–632
Reuveni M, Colombo R, Lerner HR, Pradet A, Polyakoff-Mayber A (1987) Osmotically induced proton extrusion from carrot cells in suspension culture. Plant Physiol 85:383–388
Roelfsema MRG, Hedrich R (2002) Studying guard cells in the intact plant: modulation of stomatal movement by apoplastic factors. New Phytol 153:425–431
Roos W (2000) Ion mapping in plant cells—methods and applications in signal transduction research. Planta 210:347–370
Ryan PR, Newman IA, Shields B (1990) Ion fluxes in corn roots measured by microelectrodes with ion-specific liquid membranes. J Membrane Sci 53:59–69
Ryan PR, Delhaize E, Randall PJ (1995) Characterization of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. Planta 196:103–110
Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560
Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell 11:691–706
Santos CLV, Campos A, Azevedo H, Caldeira G (2001) In situ and in vitro senescence induced by KCl stress: nutritional imbalance, lipid peroxidation and antioxidant metabolism. J Exp Bot 52:351–360
Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37:645–653
Schroeder JI, Ward JM, Gassmann W (1994) Perspectives on the physiology and structure of inward-rectifying K^+ channels in higher plants—biophysical implications for K^+ uptake. Annu Rev Biophys Biomol Struct 23:441–471
Schroeder JI, Kwak JM, Allen GJ (2001) Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature 410:327–330
Serrano R, Culianz-Macia FA, Moreno V (1999a) Genetic engineering of salt and drought tolerance with yeast regulatory genes. Sci Hort 78:261–269
Serrano R, Mulet JM, Rios G, Marquez JA, de Larrinoa IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft M, Ros R, Montesinos C (1999b) A glimpse of the mechanism of ion homeostasis during salt stress. J Exp Bot 50:1023–1036
Shabala S (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. Plant Cell Environ 23:825–837
Shabala S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. Ann Bot 92:627–634
Shabala S, Lew RR (2002) Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. Plant Physiol 129:290–299
Shabala S, Newman IA (1997) H^+ flux kinetics around plant roots after short-term exposure to low temperature—identifying critical temperatures for plant chilling tolerance. Plant Cell Environ 20:1401–1410
Shabala S, Newman IA (1998) Osmotic sensitivity of Ca^{2+} and H^+ transporters in corn roots—effect on fluxes and their oscillations in the elongation region. J Membrane Biol 161:45–54
Shabala S, Newman IA (1999) Light-induced changes in hydrogen, calcium, potassium, and chloride ion fluxes and concentrations from the mesophyll and epidermal tissues of bean leaves. Understanding the ionic basis of light-induced bioelectrogenesis. Plant Physiol 119:1115–1124
Shabala S, Shabala L (2002) Kinetics of net H^+, Ca^{2+}, K^+, Na^+, NH_4^+, and Cl^- fluxes associated with post-chilling recovery of plasma membrane transporters in *Zea mays* leaf and root tissues. Physiol Plant 114:47–56
Shabala S, Newman IA, Morris J (1997) Oscillations in H^+ and Ca^{2+} ion fluxes around the elongation region of corn roots and effects of external pH. Plant Physiol 113:111–118
Shabala S, Newman I, Whittington J, Juswono U (1998) Protoplast ion fluxes: their measurement and variation with time, position and osmoticum. Planta 204:146–152
Shabala S, Babourina O, Newman I (2000) Ion-specific mechanisms of osmoregulation in bean mesophyll cells. J Exp Bot 51:1243–1253

Shabala L, Ross T, Newman I, McMeekin T, Shabala S (2001a) Measurements of net fluxes and extracellular changes of H^+, Ca^{2+}, K^+, and NH_4^+ in *Escherichia coli* using ion-selective microelectrodes. J Microbiol Method 46:119–129

Shabala L, Shabala S, Ross T, McMeekin T (2001b) Membrane transport activity and ultradian ion flux oscillations associated with cell cycle of *Thraustochytrium sp*. Aust J Plant Physiol 28:87–99

Shabala L, Budde B, Ross T, Siegumfeldt H, Jakobsen M, McMeekin T (2002) Responses of *Listeria monocytogenes* to acid stress and glucose availability revealed by a novel combination of fluorescence microscopy and microelectrode ion-selective techniques. Appl Environ Microbiol 68:1794–1802

Shabala S, Shabala L, van Volkenburgh E (2003) Effect of calcium on root development and root ion fluxes in salinised barley seedlings. Funct Plant Biol 30:507–514

Shabala L, Ross T, McMeekin T, Shabala S (2005a) Non-invasive microelectrode ion flux measurements to study adaptive responses of surface associated microorganisms to the environment. FEMS Microbial Rev (in press)

Shabala S, Shabala L, van Volkenburgh E, Newman I (2005b) Effect of divalent cations on ion fluxes and leaf photochemistry in salinised barley leaves. J Exp Botany 56:1369–1378

Shabala L, Cuin TA, Newman I, Shabala S (2005c) Salinity-induced ion flux patterns from the excised roots of *Arabidopsis sos* mutants. Planta (published on-line first DOI: 10.1007/s00425–005–0074–2)

Shipley AM, Feijo JA (1999) The use of the vibrating probe technique to study steady extracellular currents during pollen germination and tube growth. In: Cresti M, Cai G, Moscatelli A (eds) Fertilization in higher plants: molecular and cytological aspects. Springer, Berlin Heidelberg New York, pp 235–250

Shorning BY, Smirnova EG, Yaguzhinsky LS, Vanyushin BF (2000) Necessity of superoxide production for development of etiolated wheat seedlings. Biochemistry (Moscow) 65:1357–1361

Smethurst CF, Shabala S (2003) Screening methods for waterlogging tolerance in lucerne: comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. Funct Plant Biol 30:335–343

Smirnoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Plant Sci 19:267–290

Smith PJS (1995) Non-invasive ion probes—tools for measuring transmembrane ion flux. Nature 378:645–646

Spalding EP (2000) Ion channels and the transduction of light signals. Plant Cell Environ 23:665–674

Subbaiah CC, Bush DS, Sachs MM (1998) Mitochondrial contribution to the anoxic Ca^{2+} signal in maize suspension-cultured cells. Plant Physiol 118:759–771

Suhita D, Raghavendra AS, Kwak JM, Vavasseur A (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol 134:1536–1545

Sutton JM, Lea EJA, Downie JA (1994) The nodulation-signaling protein *nodo* from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. Proc Natl Acad Sci USA 91:9990–9994

Tegg RS, Melian L, Wilson CR, Shabala S (2005) Plant cell growth and ion flux responses to the *Streptomycete* phytotoxin thaxtomin-A: calcium and hydrogen flux patterns revealed by the non-invasive MIFE technique. Plant Cell Physiol 46:638–648

Teodoro AE, Zingarelli L, Lado P (1998) Early changes of Cl^- efflux and H^+ extrusion induced by osmotic stress in *Arabidopsis thaliana* cells. Physiol Plant 102:29–37

Tester M (1997) Techniques for studying ion channels: an introduction. J Exp Bot 48:353–359

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503–527

Tomos AD, Leigh RA (1999) The pressure probe: a versatile tool in plant cell physiology. Annu Rev Plant Physiol Plant Mol Biol 50:447–472

Trono D, Flagella Z, Laus MN, Di Fonzo N, Pastore D (2004) The uncoupling protein and the potassium channel are activated by hyperosmotic stress in mitochondria from durum wheat seedlings. Plant Cell Environ 27:437–448

Tyerman SD, Skerrett M, Garrill A, Findlay GP, Leigh RA (1997) Pathways for the permeation of Na^+ and Cl^- into protoplasts derived from the cortex of wheat roots. J Exp Bot 48:459–480

Tyerman SD, Beilby M, Whittington J, Juswono U, Newman I, Shabala S (2001) Oscillations in proton transport revealed from simultaneous measurements of net current and net proton fluxes from isolated root protoplasts: MIFE meets patch-clamp. Aust J Plant Physiol 28:591–604

Vera-Estrella R, Barkla BJ, Higgins VJ, Blumwald E (1994) Plant defence response to fungal pathogens. Activation of host-plasma membrane H^+-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol 104:209–215

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O (1999) Salt stress in *Mesembryanthemum crystallinum* L cell suspensions activates adaptive mechanisms similar to those observed in the whole plant. Planta 207: 426–435

Very AA, Sentenac H (2002) Cation channels in the *Arabidopsis* plasma membrane. Trend Plant Sci 7:168–175

Wang MY, Siddiqi MY, Ruth TJ, Glass ADM (1993) Ammonium uptake by rice roots. II. Kinetics of $^{13}NH_4^+$ influx across the plasmalemma. Plant Physiol 103:1259–1267

Ward JM (2001) Identification of novel families of membrane proteins from the model plant *Arabidopsis thaliana*. Bioinformatics 17:560–563

Wherrett T, Ryan P, Delhaize E, Shabala S (2005) Effect of aluminium on membrane potential and ion fluxes at the apices of wheat roots. Funct Plant Biol 32:199–208

White PJ, Clarkson DT, Earnshaw MJ (1987) Acclimation of potassium influx in rye (*Secale cereale*) at low root temperatures. Planta 171:377–385

Wyn Jones RG, Pritchard J (1989) Stresses, membranes and cell walls. In: Jones HG, Flowers TJ, Jones MB (eds) Plants under stress: biochemistry, physiology, and ecology and their application to plant improvement. Cambridge University Press, Cambridge, pp 95–114

Xia JH, Roberts JKM (1996) Regulation of H^+ extrusion and cytoplasmic pH in maize root tips acclimated to a low-oxygen environment. Plant Physiol 111:227–233

Yoshida S (1991) Chilling-induced inactivation and its recovery of tonoplast H^+-ATPase in mung bean cell suspension cultures. Plant Physiol 95:456–460

Yu L, Moshelion M, Moran N (2001) Extracellular protons inhibit the activity of inward-rectifying potassium channels in the motor cells of *Samanea saman* pulvini. Plant Physiol 127:1310–1322

Zhang Q, Greenway H (1995) Membrane transport in anoxic rice coleoptiles and storage tissues of beetroot. Aust J Plant Physiol 22:965–975

Zhang Q, Lauchli A, Greenway H (1992) Effects of anoxia on solute loss from beetroot storage tissue. J Exp Bot 43:897–905

Zhang X, Miao YC, An GY, Zhou Y, Shangguan ZP, Gao JF, Song CP (2001) K^+ channels inhibited by hydrogen peroxide mediate abscisic acid signaling in *Vicia* guard cells. Cell Res 11:195–202

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445

Zimmermann S, Nürnberger T, Frachisse JM, Wirtz W, Guern J, Hedrich R, Scheel D (1997) Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defence. Proc Natl Acad Sci USA 94:2751–2755

Zimmermann S, Frachisse JM, Thomine S, Barbier-Brygoo H, Guern J (1998) Elicitor-induced chloride efflux and anion channels in tobacco cell suspensions. Plant Physiol Biochem 36:665–674

Zimmermann S, Ehrhardt T, Plesch G, Muller-Rober B (1999) Ion channels in plant signalling. Cell Mol Life Sci 55:183–203

Zingarelli L, Marre MT, Massardi F, Lado P (1999) Effects of hyper-osmotic stress on K^+ fluxes, H^+ extrusion, transmembrane electric potential difference and comparison with the effects of fusicoccin. Physiol Plant 106:287–295

Zsoldos F, Vashegyi A, Pecsvaradi A, Bona L (2001) Growth and potassium transport in common and durum wheat as affected by aluminum and nitrite stress. J Plant Nutr 24:345–356