# Chapter 4
# Application of Non-invasive Microelectrode Flux Measurements in Plant Stress Physiology

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**Abstract** Non-invasive microelectrode flux measurement (the MIFE$^{TM}$ technique) is a convenient tool to study membrane-transport processes in plants in situ. Over the last 20 years, many papers have been published elucidating the critical role of membrane-transport processes in response to a variety of abiotic and biotic stresses including salinity, osmotic stress, temperature extremes, acidity, oxygen deprivation, nutritional disorders, oxidative stress, and pathogens and elicitors. In this review, we summarize some of these findings and illustrate how the application of ion-selective microelectrodes may be combined with other techniques to address some fundamental issues related to mechanisms of plant nutrient acquisition and stress signaling and adaptation.

**Abbreviations**

| | |
|---|---|
| *abi1* | Abscisic acid-insensitive1 |
| *akt1* | *Arabidopsis* $K^+$ transporter1 |
| ALMT | Aluminum-activated malate transporter |
| AM | Arbuscular mycorrhiza |
| AtCNGC10 | *Arabidopsis* cyclic nucleotide-gated channel10 |
| *AtHELPS* | *Arabidopsis* DExD/H box RNA helicase |
| ATP | Adenosine triposphate |
| CED-9 | Cell death defective-9 |
| Cu/a | $Cu^{2+}$/ascorbate |
| $[Ca^{2+}]_{cyt}$ | Cytosolic free calcium |
| Em | Membrane potential |
| GORK | Guard cell outward-rectifying $K^+$ channel |
| HR | Hypersensitive response |

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| | |
|---|---|
| Kcv | Viral-encoded $K^+$ channel |
| KIR | $K^+$ inward-rectifying channel |
| KOR | $K^+$ outward-rectifying channel |
| LIX | Liquid ion exchanger |
| MATE | Multidrug and toxic efflux transporter |
| MBS | Marine bioactive substances |
| NORC | Nonspecific outward-rectifying channel |
| NSCC | Non-selective cation channel |
| $O_2$ | Oxygen |
| PAMPs | Pathogen-associated molecular patterns |
| PA | Polyamines |
| PBCV-1 | *Paramecium bursaria* chlorella virus |
| PCD | Programmed cell death |
| PM | Plasma membrane |
| PMV | Papaya mosaic virus |
| PVX | Potato virus X |
| ROS | Reactive oxygen species |
| SAC | Stretch-activated channels |
| SIET | Scanning ion-selective electrode technique |
| TEA | Tetraethylammonium |
| TUNEL | Terminal deoxynucleotidyl dUTP nick end labeling |
| *ucu2-2/gi2* | *ultracurvata2-2/gigantea-2* |
| UV-C | Ultraviolet-C |
| zVAD-fmk | Benzyloxycarbonyl-Val-Ala-Asp (OMe)—uoromethylketone |

## 4.1 Introduction

The idea of measuring a specific ion flux non-invasively using ion-selective microelectrodes that move between two positions near the tissue was proposed as early as in 1985 (Lucas and Kochian 1986). Following the initial development stage (Newman et al. 1987; Kochian et al. 1989), two automated systems were developed for computer-controlled electrode movement and data recording, independently at the University of Tasmania and at the MBL (Marine Biological Laboratory, Woods Hole, MA)(Newman et al. 2012). These two systems are now known as MIFE$^{TM}$ (Microelectrode ion flux measurement) and SIET (Scanning ion-selective electrode technique), accordingly.

Since the mid-90s our laboratory has pioneered application of non-invasive ion flux measuring (the MIFE technique) in plant stress physiology. This technique provides an impressive capability to link genetic/genomic data to cellular physiological behavior, thus providing a valuable contribution to functional genome/phenome research. Over

100 papers have been published by both us and other researchers using MIFE (or its equivalent) technique for elucidating the critical role of membrane-transport processes in response to a variety of abiotic and biotic stresses including salinity, osmotic stress, temperature extremes, acidity, oxygen deprivation, nutritional disorders, oxidative stress, and pathogens and elicitors. In this review, we summarize some of these findings and illustrate how the application of ion-selective microelectrodes may be combined with other techniques to address some fundamental issues related to mechanisms of plant nutrient acquisition and stress signaling and adaptation.

## 4.2 Nutrient Acquisition and Transport

The non-invasive vibrating microelectrode technique has been used widely to decipher nutrient deficiency, acquisition, and transport mechanisms in plants. The following section summaries pilot studies undertaken in this area of research.

Upon iron (Fe) limitation, plants use two strategies to improve Fe acquisition: Strategy I plants (all plants except grasses) upregulate $H^+$-ATPase ($H^+$ efflux) in order to acidify the rhizosphere thereby increasing solubility/reduction of Fe in the rhizosphere, and strategy II plants excrete phytosiderphores which form stable Fe-chelates for transport into the plant (Walker and Connolly 2008). Rice presents a special case: being a strategy II plant, it also possess a strategy I mechanism to take up reduced Fe(II) from flooded-hypoxic environment. In this regard, a recent study isolated a vesicle-related protein OsSEC27P from rice and demonstrated enhanced $H^+$ secretion in transgenic tobacco roots under Fe deficiency conditions (Yang et al. 2010).

Magnesium(Mg) deficiency-induced inhibition of photosynthesis is a widely accepted phenomenon. However, such inhibition occurs only after prolonged growth (few weeks to months) in Mg-deficient media. Thus early detection is critical for adopting corrective measures. In this regard, Hariadi and Shabala (2004) found that light-induced changes in leaf surface electric potential can be used as the early (within two weeks of emergence) detection tool for Mg-deficiency, with a 2-fold difference in the magnitude of leaf bioelectric response between Mg-deficient (10 ppm) and optimal (50 ppm) treatments. This difference in leaf surface electric potential was explained by the difference in $Mg^{2+}$ movement across the plasma membrane. Subsequent analysis of $Mg^{2+}$ transport revealed that two kinds of $Mg^{2+}$ transporters viz., $Mg^{2+}/H^+$ exchanger (at $\leq$30 μM range) and non-selective cation channel (>300 μM range) were involved in $Mg^{2+}$ uptake across the plasma membrane of bean mesophyll cells (Shabala and Hariadi 2005).

Nodulation and mycorrhizal symbiosis are the effective strategies employed by the plants to fix atmospheric nitrogen and increase soil nutrient uptake, respectively. Though a number of anatomical (e.g. increase in the nutrient absorbing area) and physiological mechanisms (e.g. synthesis and exudation of organic compounds, exoenzymes to solubilize nutrients) are clearly involved, the exact mechanism by which host recognition and enhanced nutrient uptake are achieved is poorly understood (Ramos et al. 2009). To shed light on this issue, Ramos et al. (2008) traced the

pre-symbiotic development of the arbuscular mycorrhizal (AM) fungi, and found that $H^+$ flux "signature" was involved in host recognition. Particularly, AM fungi showed increase in $H^+$ efflux and hyphal growth rate when the fungus was growing close to clover roots or was pre-treated with root exudates (Ramos et al. 2008). In a separate study, ectomycorrhizal roots of *Eucalyptus* showed 6-fold increase in $H^+$ efflux and concomitant rhizophere acidification at the elongation zone (Ramos et al. 2009). Similarly, Ding et al. (2011) inoculated soybean with nodule forming rhizobia and AM fungus and observed 3 to 8-fold increase in $H^+$ efflux during synergistic interaction in the plant-nodules-hyphae-rhizosphere continuum. Thus, elevated $H^+$ efflux and accompanied rhizophere acidification is the key determinant for enhanced nutrient uptake during nodule and/or symbiosis formation.

Soil or foliar application of marine bioactive substances (MBS; a biostimulant) extracted from sea weed received considerable attention, due to their potential use in organic and sustainable agriculture as a means to avoid excessive fertilizer application and to improve mineral absorption (Mugnai et al. 2008). It is believed that MBS improves crop growth through the enhanced supply of major and minor nutrients, amino acids, vitamins, and also cytokinins, auxin, and absicisic acid-like growth substances (see Mugnai et al. 2008). Recent studies from Mancuso's laboratory showed that MBS enhance $NH_4^+$, $K^+$, and $Ca^{2+}$ uptake into *Vitis vinifera* roots, with beneficial effects for plant growth (Mancuso et al. 2006; Mugnai et al. 2008).

Based on anatomy and growth pattern, plant roots are divided into five distinct zones along the longitudinal axis viz., root cap, meristem (zone of active cell division), distal elongation zone or transition zone (zone of slow cell growth in length and width), elongation zone (zone of rapid cell growth in length without growth in width), and mature zone (zone of root hairs) (Verbelen et al. 2006). It is hypothesized that these root zones may differ distinctly in nutrient acquisition and plant signaling in response to environmental stimuli. From this perspective, non-invasive vibrating microelectrodes form the only technique available to provide the required spatial resolution along the longitudinal axis. It has been demonstrated now that $H^+$, $K^+$ (Bose et al. 2010b), $NH_4^+$, $NO_3^-$ (Hawkins et al. 2008), $Ca^{2+}$, and $Mg^{2+}$ (Guo et al. 2010) fluxes differ between different root zones. Moreover, this technique can be used to do functional analysis for individual transporters at the molecular level. For example, (Guo et al. (2010) demonstrated that *AtCNGC*10 transporter (*Arabidopsis* cyclic nucleotide-gated channel) is involved in transporting $Ca^{2+}$ and $Mg^{2+}$ ions. Similarly, functional analysis of *AtHELPS*, an *Arabidopsis* DExD/H box RNA helicase revealed that *AtHELPS* is a negative regulator of high affinity $K^+$ transporters (e.g. *AKT1- Arabidopsis* $K^+$ *transporter1*), required during $K^+$ deprivation (Xu et al. 2011).

## 4.3 Salinity

Salinity is a major environmental problem affecting crop production around the world, with up to 7% of the total land surface on earth being saline (Flowers and Yeo 1995). The economic penalties are in the range of billions of dollars. To tackle

the problem, large-scale soil amelioration has to be complemented by efficient breeding programs to increase the salt tolerance of plants, by either traditional breeding or genetic manipulation technologies (Tester and Davenport 2003).

Salt tolerance in plants is conferred by a large number of adaptive mechanisms, most of which are related to membrane-transport processes. Not surprisingly, application of the MIFE technique has allowed us to address many of the fundamental issues related to salinity stress signaling and tolerance in both glycophyte and halophyte species.

### 4.3.1 Revealing Osmotic and Na-Specific Components of Salt Stress Signaling

The two principal adverse effects of salinity in non-tolerant plants are osmotic stress and specific ion ($Na^+$ or $Cl^-$) toxicity (Zhu 2003; Tester and Davenport 2003). We have shown that the ionic basis of plant adaptive responses to each of these components of salinity is strikingly different (Shabala 2000; Chen et al. 2005). While NaCl promotes a net $K^+$ efflux, isotonic mannitol treatment induces a gradual increase in the net $K^+$ uptake leading to turgor recovery. This difference is explained by the specificity of effects of "ionic" and "osmotic" components of the salt stress on cell membrane potential, affecting $K^+$ transport via voltage-gated inward- and outward-rectifying $K^+$ channels (Shabala and Cuin 2008). $K^+$ efflux is mediated essentially by depolarization-activated $K^+$ outward-rectifying channels (KOR), while inward-rectifying channels (KIR) are responsible for $K^+$ uptake in response to non-ionic hyperosmotic treatment. Thus, two oppositely directed signals appear to initiate $K^+$ fluxes from salinity-stressed plant cells: (1) $K^+$ efflux resulting from NaCl-induced plasma membrane depolarization, and (2) $K^+$ uptake resulting from an as yet unknown "osmosensing mechanism". Under mild salinities, the latter component would dominate, while at higher NaCl levels, the result would be a net loss of $K^+$.

### 4.3.2 Revealing Essentiality of Cytosolic $K^+$ Retention as a Key Determinant of Plant Salinity Tolerance

$Na^+$ toxicity occurs as a result of its competition with $K^+$ for enzyme activation and protein biosynthesis. From this point of view, it is not the absolute quantity of $Na^+$ per se, but rather the cytosolic $K^+/Na^+$ ratio that determines cell metabolic competence and ultimately, the ability of a plant to survive in saline environments (Shabala and Cuin 2008). Thus, efficient cytosolic $K^+$ retention is absolutely essential for plant salinity tolerance. A strong positive correlation between shoot $K^+$ concentration and genotype's salinity tolerance was reported for a wide range

of plant species (Cuin et al. 2003; Colmer et al. 2006; Chen et al. 2005, 2007; Cuin et al. 2010).

Of 75 $K^+$-permeable transporters found in plants (Very and Sentenac 2002; Shabala 2003), depolarization-activated outward-rectifying $K^+$ (KOR) channel and non-selective cation (NSCC) channel play the dominant role in maintaining the optimal cytosolic $K^+$ homeostasis and controlling salinity-induced $K^+$ leak (Shabala and Cuin 2008). Under saline conditions, the root plasma membrane is strongly depolarized as a consequence of a massive influx of positively charged $Na^+$ ions. This makes $K^+$ uptake through KIR channels thermodynamically impossible, and plants must rely exclusively on $K^+$ uptake via high affinity transport systems (which is much less efficient). Even more importantly, the observed depolarization not only makes $K^+$ uptake more problematic but also causes a massive $K^+$ efflux through KOR channels. Taken together, these two factors result in a massive depletion in the cytosolic $K^+$ pool (Cuin et al. 2003; Shabala et al. 2006).

The potassium retention trait seems to be highly heritable (Chen et al. 2005, 2008; Cuin et al. 2011b), opening prospects of using MIFE for plant seedling screening in breeding programs. Simple protocols have been developed to screen plant accessions for salinity tolerance in this species (Chen et al. 2005, 2007b). These could be used by plant breeders in order to achieve their aim of developing salinity tolerant crop varieties.

### *4.3.3 Resolving the Role of the Plasma Membrane $H^+$-Pump in Salinity Responses*

A salinity-induced increase in $H^+$-pump activity can provide a driving force for a plasma membrane $Na^+/H^+$ exchanger to move $Na^+$ from the cytoplasm into the apoplast and has been reported in many halophytic species (Ayala et al. 1996; Vera-Estrella et al. 1999; Vera-Estrella et al. 2005). However, in *Aster tripolium* NaCl-induced stimulation of P-ATPase activity was observed only after one day of salt treatment and was followed by a pronounced decline of the P-ATPase activity (Ramani et al. 2006). In *Plantago maritima*, such treatment caused a decrease in the plasma membrane $H^+$-ATPase activity in leaves (Bruggemann and Janiesch 1989). Is higher $H^+$-ATPase activity essential for salinity stress tolerance, and is there any difference between halophyte and glycophyte species?

Using the MIFE technique we have shown that NaCl application induces vanadate-sensitive $H^+$ efflux from both leaf (Shabala 2000) and root (Shabala et al. 2005b) tissues in a range of glycophyte species. Furthermore, higher $H^+$-pump activity correlated significantly with a cultivar's tolerance (Chen et al. 2007b). More tolerant varieties were able to maintain more negative membrane potential values under saline conditions and, thus, were able to prevent (or significantly reduce the magnitude of) NaCl-induced $K^+$ leak from the cytosol. With plasma membrane (PM) $H^+$-ATPase being a major determinant of membrane potential (Em; Michelet

and Boutry 1995; Palmgren 2001), more negative $E_m$ values in salt-tolerant genotypes under steady-state conditions could be a direct consequence of a more active $H^+$ pump. However, in contrast to some other species (Elkahoui et al. 2005; Yang et al. 2006), Western blot analysis revealed no difference in the amount of protein present between different cultivars (Chen et al. 2007b). This suggests that the 5-fold difference in $H^+$-ATPase activity observed between contrasting cultivars was due to post-translational modulation of the ATPase.

### 4.3.4 *Understanding Ameliorative Effects of $Ca^{2+}$ and Divalent Cations*

The application of $Ca^{2+}$ significantly ameliorates salinity stress in many species (LaHaye and Epstein 1969; Cramer et al. 1987; Reid and Smith 2000; Shabala et al. 2003). Given the fact that such amelioration is also observed in hydroponically grown plants, the beneficial effects of supplemental $Ca^{2+}$ are not related to changes in the soil structure and result from direct interaction between $Ca^{2+}$ and root ion transporters. It has traditionally been accepted that the dominating mechanism behind this was the $Ca^{2+}$ restriction of $Na^+$ uptake via non-selective cation channels (NSCC), the likely main pathway for $Na^+$ uptake into the cell (Tyerman et al. 1997; Demidchik and Tester 2002). Using MIFE, we have shown that supplemental $Ca^{2+}$ also efficiently reduces or even prevents NaCl-induced $K^+$ efflux through GORK (Guard cell outward-rectifying $K^+$ channel) channels (Shabala et al. 2003, 2005b, 2006). The concurrent blockage by supplemental $Ca^{2+}$ of both $Na^+$ uptake via NSCC and $K^+$ loss via efflux channels will have beneficial effect on the cytosolic K/Na ratio and cell metabolism under saline conditions. Importantly, not only $Ca^{2+}$ but other divalent cations such as $Mg^{2+}$ and $Ba^{2+}$ were capable of inhibiting $Na^+$-induced $K^+$ efflux (Shabala et al. 2005b), and these effects were observed not only in root but also in shoot tissues.

### 4.3.5 *Elucidating the Role of Polyamines in Plant Adaptive Responses to Salinity*

Polyamines (PAs; putrescine, spermidine, and spermine) are plant growth regulators, critical for a number of developmental processes, including cell division, somatic embryogenesis, root growth, floral initiation, and flower and fruit development (Evans and Malmberg 1989; Galston and Kaur-Sawhney 1990). In addition to their role in plant development, PA may also play an important role in plant stress responses and, specifically, responses to salinity. A positive correlation between the level of unconjugated polyamines and plant salinity tolerance has been reported (Basu and Ghosh 1991; Erdei et al. 1990)

The specific details of how PA may mediate plant adaptation to salinity remain elusive. Reduced $Na^+$ accumulation in leaves and higher $K^+/Na^+$ ratios in the shoot of PA treated plants grown in the presence of NaCl have been reported at the whole-plant level (Lakra et al. 2006; Ndayiragije and Lutts, 2006). This phenomenon was explained by the ability of PA to control permeability of both non-selective, NSCC (Shabala et al. 2007a) and outward-rectifying $K^+$ (Pandolfi et al. 2010) channels and, hence, directly affect the intracellular $K^+/Na^+$ ratio. Indirect effects are also possible. First, PA blockage of NSCC will restrict inward $Na^+$ flux and a subsequent membrane depolarization, reducing NaCl-induced $K^+$ leak from the cytosol. Second, PA may regulate the activity of plant $H^+$-ATPases through enhancing their interaction with 14-3-3 proteins (Garufi et al. 2007), also affecting plasma membrane potential and, by doing this, channel permeability.

PAs have been also shown to possess the ability to influence reactive oxygen species (ROS) scavenging. This may be achieved directly, via increasing the activities of antioxidant systems, both enzymatic (APX, SOD) and non-enzymatic (glutathione and carotenoids) (Tang and Newton 2005; Verma and Mishra 2005). Indirect control is also very likely. Very recently, we have shown that PAs act as cofactors in the ROS-induction of PM $Ca^{2+}$ -permeable ion channels and can also induce active $Ca^{2+}$ efflux from the cell by activating PM-based $Ca^{2+}$-ATPase (Zepeda-Jazo et al. 2011).

### *4.3.6 Revealing Mechanisms of Ion Loading into the Xylem*

Among the multiple physiological mechanisms contributing to plant salinity tolerance, reducing $Na^+$ loading into the xylem is often named as one of the most crucial features (Tester and Davenport 2003; Munns and Tester 2008). This can be achieved either by minimization of $Na^+$ entry to the xylem from the root symplast, or by maximization of retrieval back out from the xylem before it reaches sensitive tissues in the shoot.

Application of a range of biophysical and physiological kinetics of $Na^+$ and $K^+$ loading into the xylem was studied in barley varieties contrasting in their salt tolerance (Shabala et al. 2010b). It was found that restricting $Na^+$ loading into the xylem is not essential for conferring salinity tolerance in barley, with tolerant varieties showing xylem $Na^+$ concentrations at least as high as sensitive ones. At the same time, tolerant genotypes were capable of maintaining higher xylem $K^+/Na^+$ ratios and efficiently sequestering the accumulated $Na^+$ in leaves (Shabala et al. 2010b). The former was achieved by more efficient loading of $K^+$ into the xylem, and evidence was presented that $K^+$-permeable voltage-sensitive channels in parenchyma cells are involved in xylem loading and that they operate in a feedback manner to maintain a constant $K^+/Na^+$ ratio in the xylem sap. As for $Na^+$ loading, two possibilities exist: (1) passive loading mediated by $Na^+$-permeable ion channels located at the xylem-parenchyma interface, or (2) active loading mediated by SOS1 $Na^+/H^+$ exchangers (Shabala 2007).

Both MIFE measurements on isolated stellar tissues (Shabala et al. 2010b) and *in planta* measurements using multifunctional xylem pressure probes (Wegner et al. 2011) favor an active $Na^+$ loading concept. The most likely candidate for this active loading is the $Na^+/H^+$ antiporter SOS1. Such antiporters were shown to be preferentially expressed at the xylem symplast boundary of roots (Shi et al. 2002) and use $H^+$-ATPase energy to pump $Na^+$ against its electrochemical gradient existing between the parenchyma cell cytosol and the xylem (Shabala and Mackay 2011). When cytosolic $Na^+$ content in xylem parenchyma cells becomes higher (e.g. above 100 mM as was suggested in some works; e.g. Flowers and Colmer 2008), and xylem $Na^+$ content is low, NORC (Nonspecific outward-rectifying) channels (Wegner and Raschke 1994) may be responsible for the passive $Na^+$ loading into the xylem. However, such a situation is likely to happen only after very prolonged NaCl exposures. In all other cases, plants favor active $Na^+$ loading to achieve quick osmotic adjustment in the shoot.

### 4.3.7 *Quantifying Kinetics of $Na^+$ Transport in Salinity-Stressed Plants*

Measurements of net $Na^+$ fluxes from salinized plant tissues are significantly handicapped by two factors. One is the unfavorable flux signal to noise ratio observed under saline conditions; the other one is poor selectivity of all available commercial $Na^+$ LIX (Liquid ion exchanger; Carden et al. 2003; Chen et al. 2005). The latter problem is manifested by the fact that $Na^+$ LIX has an almost ideal Nernst response when calibrated in a set of $K^+$ or $Ca^{2+}$ standards (Chen et al. 2005). Thus, any apparent $Na^+$ flux recorded after the imposition of NaCl is confounded by the massive efflux of $K^+$ (see above) and $Ca^{2+}$ (the result of Donnan exchange in the cell wall; Ryan et al. 1992; Shabala and Newman 2000) that occur in response to salinity treatment. In consequence, the net $Na^+$ uptake will be substantially underestimated.

To overcome the above limitations, a new method defined as a "recovery protocol" was developed (Cuin et al. 2011a) involving recording the net efflux of $Na^+$ in a $Na^+$-free medium, immediately after the removal of NaCl. This method was successfully used to show that a more salinity tolerant wheat variety has a far superior ability to export $Na^+$ out of the plant root than more susceptible varieties (Cuin et al. 2011a). Pharmacological experiments, as well as experiments on a range of $Na^+$ transport mutants, have revealed that this flux is mediated by the SOS1-like $Na^+/H^+$ antiporters located in root epidermal cells. Evidence for high inheritance of this trait has been presented (Cuin et al. 2011b). Thus, the developed MIFE protocols provide a relatively reliable and straightforward method for rapidly assessing a plant's ability to actively export $Na^+$ and, thus, can be used in breeding programs to select varieties with high $Na^+$ extruding capacity.

## 4.4 Osmotic Stress

### *4.4.1 Osmotic Adjustment in Plants*

Osmotic stress is ubiquitous in nature and can be manifested by a range of environmental conditions such as drought or salinity. It results in severe disturbance to cell metabolism, plant growth and survival and severely limits global agricultural productivity, resulting in multibillion dollar penalties. To adjust to increased external osmolality, cells in all three kingdoms accumulate a variety of molecules in the cytoplasm to counteract the external osmotic pressure. Two major avenues are available for plants.

First, cells can achieve osmotic adjustment by accumulation (de novo synthesis) of so-called *compatible solutes*—small water-soluble molecules that may be accumulated in cells at high concentrations without affecting metabolic reactions in either the cytosol or major organelles (Hasegawa et al. 2000). Four major classes of osmolytes are usually distinguished (Delauney and Verma 1993): (1) sugars (e.g. sucrose or trehalose); (2) polyoles (e.g. glycerol, sorbitol, or mannitol); (3) amino acids (e.g. glutamate or proline); (4) quaternary ammonium compounds (e.g. glycine betaine). According to the classical view, accumulation of these non-toxic (thus *compatible*) osmotically active solutes will result in an increase in cellular osmolarity leading to the influx of water into, or at least reduced efflux from, cells, thus providing the turgor necessary for cell expansion (Delauney and Verma 1993; Hare et al. 2002).

### *4.4.2 Are Compatible Solutes Actually Involved into Osmotic Adjustment?*

The above concept became a dogma and has dominated the literature for the last two decades (Shabala and Shabala 2011). Improving crop resistance to osmotic stresses by overexpressing genes responsible for biosynthesis of various compatible solutes has long been an attractive and widely popular option (Bohnert et al. 1995; Bray 1997; Bohnert and Shen 1999; Serrano et al. 1999; Bajaj et al. 1999). However, despite all efforts, the progress is disappointingly slow, and an increase in the osmotic tolerance in a field situation is only marginal (Bohnert and Shen 1999; Bajaj et al. 1999). Several major reasons may explain this:

1. Concentrations are always far too low (Williamson and Slocum 1992; Peng et al. 1996; Igarashi et al. 1997; Shen et al. 1997; Garg et al. 2002) and not enough to account for maintenance of the cell turgor under hyperosmotic conditions. As the very best, organic osmolytes may be accountable for osmotic adjustment in the cytosol and/or plastids.

2. With a possible exception for the food-dwelling microorganisms, concentration of organic osmolytes in the external environment is usually far too low to rely upon for osmotic adjustment. Hence, plants and bacteria have to synthesize these compatible solutes de novo. This comes at a very high cost to the organism, as between 30 and 109 molecules of ATP (Adinosine triposphate) appear to be required for the autotrophic biosynthesis of one molecule of the different compatible solutes (Raven 1985; Oren 1999).
3. Synthesis of compatible solutes is a rather slow process, operating in a time-scale of hours and days. At the same time, many organisms may experience much faster fluctuations in media osmolality and, hence, require more quick ways for osmotic adjustment.
4. The last and, arguably, most important controversy is the lack of consistent correlation between osmotic stress tolerance and accumulation of organic osmolytes. *Arabidopsis rss* mutant with reduced salt sensitivity showed much less capacity for proline accumulation under both salinity and osmotic stresses compared with wild type (Werner and Finkelstein 1995). Salt-sensitive genotypes often accumulate much more organic osmolytes compared with tolerant varieties (Lutts et al. 1999; Colmer et al. 2006; Chen et al. 2007a).

### *4.4.3 Inorganic Ion Uptake and Plant Osmotic Adjustment*

A viable alternative to the energetically expensive and rather slow (see above) process of osmolyte biosynthesis is osmotic adjustment by means of inorganic ions (Shabala and Shabala 2011). Indeed, from a thermodynamical point of view, water retention within the cell may be achieved equally well by increased concentration of both organic and inorganic molecules. The idea that changed ion fluxes in response to osmotic stress may provide quick (within a few minutes) osmotic adjustment and maintain normal turgor is rather old (e.g. Wyn Jones and Pritchard 1989). However, direct experimental evidence is rather scant and mostly based on theoretical calculations of changes in cell osmotic potential caused by measured fluxes of inorganic ions (Okazaki et al. 1984; Teodoro et al. 1998). The first direct support at the cellular level came from concurrent measurements of net ion fluxes in osmotically stressed *Arabidopsis thaliana* epidermal root cells (measured by the MIFE technique) and cell turgor changes (measured by the pressure-probe technique) (Shabala and Lew 2002). It was shown that over 90% of cell turgor was recovered by uptake of three major inorganic ions ($K^+$, $Na^+$, and $Cl^-$) within 40 min after onset of hyperosmotic stress. Very similar numbers were obtained for bean mesophyll cells (Shabala et al. 2000).

The above conclusions made for higher plants were later confirmed also for bacterial (Shabala et al. 2009a) and fungal (Lew et al. 2006; Shabala et al. 2009a) species. MIFE microelctrode ion flux measurements on a marine protist thraustochytrid have suggested that almost complete osmotic adjustment was achieved

in thraustochytrid cells by changes in the rate of $Na^+$, $Cl^-$, and $K^+$ fluxes within the first 30 min upon stress onset (Shabala et al. 2009b). A direct comparison between cell turgor recovery and kinetics of net ion fluxes was conducted in the filamentous fungus *Neurospora crassa* in response to hyperosmotic stress (Lew et al. 2006). The turgor recovery was completed within 60 min and occurred concurrently with net $K^+$ and $Cl^-$ uptake. The magnitude of the ion uptake was more than sufficient to account for the osmotic gradients required for turgor, ruling out a need for any organic osmolyte involvement.

### *4.4.4 On a Quest for Osmosensors*

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 by which a plant can sense osmotic conditions have been suggested by Brownlee et al. (1999). First, the changes in cell volume could be sensed by mechanosensitive, or stretch-activated, channels (SAC) at the plasma membrane (Cosgrove and Hedrich 1991; Pickard and Ding 1993; Ramahaleo et al. 1996). 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 component in ion flux oscillations measured from hyperosmotically stressed plant roots (Shabala and Newman 1998) may provide such evidence. Another option is that the intracellular osmosensing mechanisms may detect the degree of cytosol hydration (Brownlee et al. 1999). Alterations in cytoplasmic $Ca^{2+}$ concentrations were suggested as a part of the turgor signal-transduction chain for *Lamprothamnium* (Okazaki and Tazawa 1990) and *Chara* (Bisson et al. 1995).

A ubiquitous component of osmotic adjustment in higher plants is modulation of the proton-pumping activity (Reinhold et al. 1984; Reuveni et al. 1987; Li and Delrot 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. If this is the case, then osmotically induced increase in net $K^+$ uptake under hyperosmotic stress conditions caused by non-ionic osmotica (e.g. Shabala and Lew 2002; Chen et al. 2007b) can be explained by enhanced $K^+$ uptake via voltage-gated $K^+$ inward-rectifying channels or, alternatively, by reduced $K^+$ efflux through outward $K^+$ channels. Indeed, hyperosmotically induced membrane hyperpolarization has been reported in direct experiments on plant (Shabala and Lew 2002), fungal (Lew et al. 2006), and bacterial (Shabala et al. 2009a) species, and the role of inward-rectifying $K^+$ channels in root osmotic adjustment was directly shown in MIFE experiments using *Arabidopsis akt1* mutants (Shabala and Cuin 2008). It still remains, however, 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).

### 4.4.5 Multiple Roles of Organic Osmolytes

What is then the role of organic osmolytes, and why does their content increase dramatically under osmotic stress conditions? Multiple functions have been suggested, including their roles as low-molecular-weight chaperones, for membrane integrity maintenance, protecting the structure of enzymes and proteins, PSII protection and repair, redox potential buffering, and ROS scavenging (Smirnoff and Cumbes 1989; McCue and Hanson 1990; Bohnert et al. 1995; Shen et al. 1997; Hasegawa et al. 2000). Importantly, these functions do not require high amounts of organic osmolytes and, hence, do not come at high energetic cost to the organism.

Recent experiments in our laboratory have shown that compatible solutes are very efficient in reducing the extent of $K^+$ loss from cytosol in response to both salinity (Cuin and Shabala 2005, 2007a) and oxidative stress (Cuin and Shabala 2007b). Exogenously supplied physiologically relevant (5 mM) concentrations of proline and glycine betaine rapidly ameliorated NaCl-induced $K^+$ efflux from barley roots (Cuin and Shabala 2005). Further experiments have shown that 21 (of 26) protein- and non-protein- amino acids caused significant mitigation of NaCl-induced $K^+$ efflux, although two amino acids (valine and ornithine) substantially *enhanced* the detrimental effects of salinity on $K^+$ homeostasis (Cuin and Shabala 2007a). It is possible, therefore, that compatible solutes may indeed assist in plant osmotic adjustment not directly (by retaining water; which is thermodynamically impossible) but rather via retaining $K^+$. Importantly, the above mitigating effects were obtained *in situ* at physiologically relevant (0.1–1 mM) concentrations (Cuin and Shabala 2007a). Hence, plants do not need to synthesize substantial quantities of organic osmolytes to control intracellular ionic homeostasis and, ultimately, achieve osmotic adjustment via better $K^+$ retention. It should be added that, to the best of our knowledge, all previous reports of stabilizing effects of amino acids on membrane permeability and enzymatic activity were obtained in vitro and for physiologically unrealistic (e.g. 100–500 mM; Heber et al. 1971; Nash et al. 1982) concentrations. In addition, $K^+$ retention seems to be crucial for preventing ROS-induced programmed cell death (PCD; see section 4.7.3).

## 4.5 Soil pH

It is estimated that up to 70%, of the world's arable land is acidic (von Uexkull and Mutert 1995). Soil acidification is a natural process that occurs as the result of weathering of acidic parent material and leaching of basic cations. Other factors such as intensive agriculture, cultivation of legumes, use of acid-forming

fertilizers, and acid rain can also increase soil acidity. Thus, both the severity and the extent of soil acidity increase with time (Rengel 2004).

In acidic soils, plant growth may be limited by various toxicities ($H^+$, $Al^{3+}$, $Mn^{2+}$) and deficiencies ($NH_4^+$-N, P, $Ca^{2+}$, $Mg^{2+}$, and $MoO_4^{2-}$) (see Kidd and Proctor 2001). In the complex acid-soil syndrome, aluminum toxicity poses a major threat to plant growth by inhibiting root growth (Kochian et al. 2004). $Al^{3+}$ ion is the most rhizotoxic form (Kochian 1995); its activity peaks at around pH 4.2–4.3 (Kinraide 1993; Taylor et al. 2000). Therefore, $Al^{3+}$ toxicity is always studied in combination with low-pH stress ($H^+$ toxicity). While these two stresses normally occur together, the mechanisms of plant tolerance to each of these may be strikingly different. It was shown that $H^+$ toxicity causes irreversible damage to primary and lateral roots in *Arabidopsis*, with the pattern of damage being different from the one caused by $Al^{3+}$ rhizotoxicity (Koyama et al. 1995; Koyama et al. 2001). Furthermore, an *Arabidopsis* QTL analysis suggested that $Al^{3+}$ tolerance and $H^+$ tolerance are controlled by different genes (Ikka et al. 2007).

A comprehensive functional characterization of *Arabidopsis* root responses to low-pH and combined low-pH/$Al^{3+}$ stresses have been recently undertaken using the MIFE technique. In the absence of $Al^{3+}$, low-pH stress-induced $H^+$ influx thereby causing rhizosphere alkalization, while the presence of $Al^{3+}$ inhibited $H^+$ influx and resulted in lesser rhizosphere alkalinization (Bose et al. 2010b). Moreover, aluminum sensitive *Arabidopsis* mutant *als5* grew well under low-pH stress and poorly under $Al^{3+}$ stress, whereas *als3* was sensitive and *alr104* tolerant to both stresses. Ability of *Arabidopsis* mutants to alkalinize the rhizosphere and take up $H^+$ from a low-pH environment is linked to the tolerance to low-pH and combined low-pH/$Al^{3+}$ stresses (Degenhardt et al. 1998; Bose et al. 2010a). Another MIFE study compared an acid-soil tolerant conifer (*Pseudotsuga menziesii*) with the acid-soil sensitive soybean (*Glycine max*). The results proved that ability of *P. menziesii* roots to maintain higher $H^+$ efflux and $NH_4^+$ influx than *G. max* at pH 4.0 is the key trait responsible for acid-soil tolerance in the former species (Hawkins and Robbins 2010).

$Al^{3+}$ has a strong affinity for the plasma membrane surface (Akeson et al. 1989). To prevent such binding, plants evolved several $Al^{3+}$ exclusion mechanisms; the best-described one is the root exudation of low-molecular-weight organic acid anions to increase the rhizosphere pH and reduce $Al^{3+}$ solubility (Ryan et al. 2001; Kochian et al. 2004; Kochian et al. 2005). Also, organic acid anions with a large number of carboxylate groups may directly chelate $Al^{3+}$ (Kochian et al. 2004). Depending on the plant species, $Al^{3+}$ activates exudation of various organic acid anions such as malate, citrate, oxalate, pyruvate, and/or succinate (Larsen et al. 1998; Ryan et al. 2001; Kochian et al. 2005). This extrusion seems to be species-specific and mediated by organic-anion-permeable plasma membrane channels (Pineros and Kochian 2001; Zhang et al. 2001; Sasaki et al. 2004). Both aluminum-activated malate (ALMT) and citrate (MATE- Multidrug and toxic efflux) transporters have been identified in a range of plant species (reviewed in Bose et al. 2011a). Some plant species can release more than one organic acid anion in response to $Al^{3+}$ exposure (Hoekenga et al. 2006; Liu et al. 2009).

The above release of $Al^{3+}$-induced organic anions from plant roots is accompanied by $K^+$ efflux to account for electroneutrality (Ryan et al. 1995). As most studies used excised root apices and relatively short treatment times, it has remained unclear whether a similar response operates in intact roots and if it is sustained over long periods of $Al^{3+}$ exposure. Using near-isogenic wheat lines that differ in $Al^{3+}$ tolerance (ET8 and ES8), Wherrett et al. (2005) showed that addition of 50 μM $AlCl_3$ to the bathing solution stimulated an increase in $K^+$ efflux 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. These results provide new temporal and spatial information on the $Al^{3+}$-activated efflux of $K^+$ from intact wheat plants. It was further shown that membrane depolarization caused by organic-anion efflux is the mechanism behind the stimulation of $K^+$ efflux in wheat (Wherrett et al. 2005).

The action spectrum of $Al^{3+}$ appears to be much broader than just activation of anion channels. Particularly, the interaction between $Al^{3+}$ and $Ca^{2+}$ uptake received considerable attention because symptoms of severe $Al^{3+}$ toxicity resemble $Ca^{2+}$ deficiency in plants (see Foy 1988; Rengel and Elliott 1992 for references) and exogenous application of relatively high (millimolar) concentrations of $Ca^{2+}$ alleviated $Al^{3+}$ toxicity in many plant species (Brady et al. 1993; Keltjens and Tan 1993; Kinraide et al. 2004). $Al^{3+}$ might inhibit $Ca^{2+}$ influx into intact root cells (Huang et al. 1992; Ryan and Kochian 1993), protoplasts (Rengel and Elliott 1992; Rengel 1994), and membrane vesicles (Huang et al. 1996; White 1998) through binding of $Al^{3+}$ on the plasma membrane surface. Such binding of $Al^{3+}$ to the surface may block $Ca^{2+}$-permeable channels in the plasma membrane. Indeed, both the hyperpolarization-activated $Ca^{2+}$-permeable channels (Ding et al. 1993; Kiegle et al. 2000; Very and Davies 2000) and depolarization-activated $Ca^{2+}$ channels (Rengel et al. 1995; Pineros and Tester 1997) are sensitive to $Al^{3+}$. The $Ca^{2+}$ influx inhibition following $Al^{3+}$ exposure precedes root growth inhibition (Huang et al. 1992; Ryan and Kochian 1993) and, thus, is one of the potential primary causes of $Al^{3+}$ phytotoxicity in plants (Rengel 1992; Rengel and Zhang 2003). However, further studies revealed that low concentration of $Al^{3+}$ can also inhibit root growth without affecting $Ca^{2+}$ influx, and addition of ameliorating cations ($Mg^{2+}$ and $Na^+$) improved root growth, even though the net $Ca^{2+}$ influx remained inhibited (Ryan and Kochian 1993; Ryan et al. 1997). Similarly, $Al^{3+}$ caused root hair growth inhibition without affecting $Ca^{2+}$ influx in *Limnobium stoloniferum* (Jones et al. 1995). Poor correlation between Al-induced $Ca^{2+}$ influx inhibition and elongation growth of *Chara* (Reid et al. 1995) indicated that Al-induced inhibition of $Ca^{2+}$ influx alone cannot be a critical factor in triggering Al toxicity in plants.

$K^+$ is essential for cell division (Alberts et al. 1994) and turgor-dependent cell elongation. Though $K^+$ efflux during low-pH alone is well established (Babourina et al. 2001; Bose et al. 2010b), there is no causal relationship between $Al^{3+}$ toxicity and $K^+$ nutrition in plants. Both $Al^{3+}$ induced inhibition (Matsumoto and Yamaya 1986; Nichol et al. 1993) and stimulation (Lee and Pritchard 1984; Lindberg 1990; Tanoi et al. 2005) in $K^+$ uptake have been reported. A possible explanation for this

controversy could come from MIFE experiments. Future studies involving specific $K^+$ transport *Arabidopsis* mutants would pave way for identification of specific $K^+$ transporters responsible for observed phenomena during low-pH and combined low-pH/$Al^{3+}$ stress.

## 4.6 Waterlogging and Oxygen Deprivation

Waterlogging is a major constraint affecting crop growth in many agricultural regions around the world. Approximately 10% of the global land area is affected by waterlogging (Setter and Waters 2003). In Australia alone, 3.8 million ha of duplex soils in Victoria and 60% of similar soils in Western Australia experience surface and subsurface waterlogging (Greenway and Gibbs 2003). The overall loss in crop production due to waterlogging is second largest after drought (Boyer 1982). As the yield loss varies considerably (several fold) depending on the crop species, crop growth stage at which waterlogging is experienced, and stress duration (Zhou 2010), understanding the physiological mechanisms mediating plant adaptive responses to waterlogging is essential for breeding tolerant varieties.

Higher plants require continuous supply of $O_2$ to support respiration and oxidation reactions. When the soil is saturated with water, all the air from soil pore spaces is replaced by water resulting in either hypoxia or anoxia. This $O_2$ shortage for root respiration causes inhibition of root growth and decline in acquisition of major nutrients such as N, P, K, Ca, and Mg (Colmer and Greenway 2011). This form of $O_2$ deficiency is further aggravated by soil microorganisms which use residual $O_2$ in the rhizosphere (Shabala 2011). Plants usually switch from aerobic respiration to fermentation during hypoxia (low $O_2$ supply) or anoxia (absence of $O_2$) to produce ATP during waterlogging stress. Prolonged $O_2$ deprivation also results in accumulation of ethanol as the end product of fermentation and, thus, results in cytotoxicity to plants. Also, energy yield per mol of glucose is much lower (just three ATP molecules) in fermentation when compared to aerobic respiration (36 ATP molecules). As a result, plants experience significant (up to 97%; Greenway and Gibbs 2003) reduction in the rate of energy production under waterlogged conditions and, thus, have much less to invest into their growth.

Given the above importance of oxygen, it is not surprising that preventing $O_2$ loss or improving its transport to, or storage in the root, have always been central to breeding programs dealing with plant waterlogging stress tolerance. Tolerant varieties were able to maintain higher net influx of $O_2$ in the mature root zone compared with their sensitive counterparts, as revealed by $O_2$-selective microelectrode flux measurements in barley (Pang et al. 2006) and *Vitis* (Mancuso and Boselli 2002). Moreover, under anoxia stress, waterlogging tolerant *Vitis* species prevented $O_2$ loss from adventitious roots (Mancuso and Boselli 2002).

In addition to $O_2$ deficiency, accumulation of potentially toxic manganese, iron, hydrogen sulfide, various organic acids, $CO_2$, and ethylene can also pose serious

challenges to root growth in waterlogged soils (Colmer and Greenway 2011; Shabala 2011). Despite being diverse in their chemical nature, all the above factors affect membrane integrity and thus membrane transport in plants (Pang et al. 2006; Pang et al. 2007). For example, non-specific loss of $K^+$ was reported soon after onset of anoxia (Greenway et al. 1992; Colmer et al. 2001) or root exposure to secondary metabolites associated with anaerobic soils (e.g. monocarboxylic acids; (Pang et al. 2007). Thus, maintenance of membrane integrity is considered as a key factor in survival of plant cells under waterlogging stress (Rawyler et al. 1999). 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. Comparison of genotypes contrasting in waterlogging tolerance, suggested that avoiding $K^+$ loss or reducing $K^+$ leakage during hypoxia or anoxia stress is the key mechanism responsible for waterlogging tolerance in plants (Mancuso and Marras 2006; Pang et al. 2006; Pang et al. 2007; Mugnai et al. 2011). $K^+$ flux measurements along the longitudinal axis of barley roots under hypoxia revealed 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 play a vital role (Pang et al. 2006). Moreover, closure of KOR channels by rapid restoration of the membrane potential to values more negative than the $K^+$ diffusion potential may result in better $K^+$ homeostasic regulation during $O_2$ deprivation (Greenway and Gibbs 2003). Reggiani (1997) also provided evidence for cAMP-triggered KOR closure under anoxia. This is in line with a hypothesis proposed by Greenway and Gibbs (2003) that in anoxia-tolerant tissues, energy flow during anoxia must be directed toward essential nutrient transport.

Elucidating the signaling events associated with hypoxia-anoxia remains one of the great challenges. Changes in cytosolic pH (Ratcliffe 1997; Greenway and Gibbs 2003) and free calcium, $[Ca^{2+}]_{cyt}$ (Subbaiah et al. 1998) are thought to be part of the signal-transduction pathway. As cytosolic acidification was an early response observed within 5 min of anoxia (Gout et al. 2001), it was considered as the primary signal of an $O_2$ deprivation (Felle 2001). Inhibition of $H^+$-pump activity by $O_2$ deprivation was suggested as the mechanism responsible for cytoplasmic acidification (Gout et al. 2001; Tazawa 2003). However, other evidence suggests that the $H^+$-pump may in fact be upregulated (i.e. net $H^+$ extrusion increases) when cytosolic pH falls under hypoxic-anoxic conditions (Xia and Roberts 1996; Pang et al. 2006; Koizumi et al. 2011). The above controversy remains to be resolved.

Similar to cytosolic acidification, elevation of $[Ca^{2+}]_{cyt}$ is also an early response to $O_2$ deprivation. Both plasma membrane (Tamura et al. 2001; Pang et al. 2007) and endomembrane (Subbaiah and Sachs 2003a) $Ca^{2+}$ transporters seem to be involved in $[Ca^{2+}]_{cyt}$ elevation, while the resting level of $[Ca^{2+}]_{cyt}$ upon signal termination is restored by $Ca^{2+}$-ATPase (CAP1) (Subbaiah and Sachs 2003a; b).

## 4.7 Oxidative Stress

Reactive oxygen species (ROS) are produced as a by-product of cellular metabolic pathways. The major sources of ROS production are cell wall peroxidise and amine oxidase, plasma membrane NADPH oxidase, and intracellular oxidases and peroxidises in mitochondria, chloroplasts, and peroxisomes. High concentrations of ROS are detrimental to plant cells because of their ability to cause lipid peroxidation in cellular membranes, DNA damage, protein denaturation, carbohydrate oxidation, pigment breakdown, and an impairment of enzymatic activity (Noctor and Foyer 1998; Santos et al. 2001; Lee et al. 2004). However, it became increasingly clear that, in addition to being potentially hazardous products of metabolic imbalance, ROS play a very important signaling and regulatory role in plant growth, development, and adaptation. Indeed, ROS were shown to be involved in the regulation of gravitropism, stomatal aperture, cell expansion and polar growth, leaf and flower development, and programmed cell death (Cervantes 2001; Hoeberichts and Woltering 2003; Casolo et al. 2005; Wang and Song 2008). Additionally, ROS produced during abiotic stresses act to signal change and regulate gene expression (Mittler et al. 2004; Miller et al. 2008; Qiao and Fan 2008). Upstream of this signaling is ROS-induced activation of ion channels.

### *4.7.1 Revealing the Role of Compatible Solutes in Ameliorating Detrimental ROS Effects*

Detrimental effects of ROS on membrane permeability are usually attributed to non-specific effects such as oxidation of sulphydryl groups located on the ion transport proteins, peroxidation of membrane phospholipids, inhibition of membrane-bound regulatory enzymes, and disruption to oxidative phosphorylation and ATP levels (Kourie 1998). Application of a hydroxyl radical ($OH^{\bullet}$)-generating $Cu^{2+}$/ascorbate (Cu/a) mixture to plant roots results in a massive, dose-dependent efflux of $K^+$ from various plant tissues (Demidchik et al. 2003; Cuin and Shabala 2007b; Demidchik et al. 2010). The $OH^{\bullet}$-induced efflux of $K^+$ is not instantaneous but develops gradually; reaching peak values 6–15 min after treatment. In *Arabidopsis* roots, both the magnitude and time of peak $K^+$ efflux showed a strong dose-dependency on the amount of Cu/a applied (Cuin and Shabala 2007b). The $OH^{\bullet}$-induced $K^+$ efflux was sensitive to tetraethylammonium ($TEA^+$) and correlated with depolarization of the membrane potential, suggesting that it was largely mediated by depolarization-activated outward-rectifying $K^+$ channels.

Keeping in mind the requirement for strict $K^+$ homeostasis in the cell cytosol (Leigh 2001), such a massive $K^+$ efflux has a major impact on growth, metabolic performance, and survival of the plant. A large number of enzymatic and non-enzymatic antioxidants contribute to detoxication of ROS species to prevent the

above effect (Mittler et al. 2004). Among non-enzymatic antioxidants, the ability of compatible solutes to scavenge free radical species is widely reported (Smirnoff and Cumbes 1989; Bohnert et al. 1995; Shen et al. 1997; Noctor and Foyer 1998; Hong et al. 2000). Most of these results, however, were obtained from in vitro experiments. In addition, such scavenging was reported for relatively high concentrations of compatible solutes (e.g. 100 mM; Henle and Linn 1997; Shen et al. 1997; Noctor and Foyer 1998). Using the MIFE technique, we have provided the first evidence for an in situ mitigating effect of much lower (5 mM) concentrations of compatible solutes on ROS-induced ion fluxes across the plasma membrane. Interestingly, not only known free radical scavenging compatible solutes such as mannitol, myo-inositol, and proline but also glycine betaine, previously shown to be non-effective in ROS scavenging in vitro (Halliwell and Grootveld 1988; Smirnoff and Cumbes 1989) were effective in reducing the $OH^{\bullet}$-induced $K^+$ efflux. A significant difference in $OH^{\bullet}$-induced $K^+$ flux kinetics in roots pre-incubated in a range of compatible solutes and the fact that a large reduction in $OH^{\bullet}$-induced $K^+$ efflux was recorded in plants pre-incubated in glycine betaine could indicate different mechanisms of protection, such as direct transporter protection or a channel blocking role, in addition to, or as an alternative to ROS scavenging.

### 4.7.2 *Revealing Identity and Roles of ROS-Activated Cation Channels in Plant Roots*

In addition to causing massive $K^+$ efflux across the plasma membrane of *Arabidopsis* root cells, $OH^{\bullet}$ application also induced rapid $Ca^{2+}$ influx into root epidermis (Demidchik et al. 2003). This effect was mediated by ROS control upon the activity of at least two different types of plasma membrane channels. While 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), ROS-induced $K^+$ efflux was due to $OH^{\bullet}$ stimulation of an outward-rectifying potassium (KOR) channel. Experiments with *abi1* (*Abcisic acid-insensitive1*) mutant suggested that the phosphorylation state is critical to such KOR activation.

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, 2005a), acidity (Babourina et al. 2001), chilling (Shabala and Shabala 2002), and hypoxia (Pang et al. 2006). Traditionally, these effects were attributed to membrane depolarization (Shabala et al. 2003). The above finding of ROS-induced $K^+$ efflux from plant roots demonstrates that stress-induced $K^+$ efflux can be mediated by a previously unknown mechanism–activation of KOR by $OH^{\bullet}$. $K^+$ 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 responses to ROS treatment varied between species, suggesting species-specific

"flux signatures" in response to $OH^{\bullet}$ (Demidchik et al. 2003). 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 *vs* 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).

We have also shown that not only $OH^{\bullet}$ but also exogenous $H_2O_2$ application to *A. thaliana* root epidermis results in dose-dependent transient increases in net $Ca^{2+}$ influx (Demidchik et al. 2007). The magnitude and duration of the transients were greater in the elongation zone than in the mature epidermis at all concentrations tested (10 μM to 10 mM). Application of 10 mM $H_2O_2$ to the external plasma membrane face of elongation zone epidermal protoplasts resulted in the appearance of a hyperpolarization-activated $Ca^{2+}$-permeable conductance. In contrast, in mature epidermal protoplasts a plasma membrane hyperpolarization-activated $Ca^{2+}$-permeable channel was activated only when $H_2O_2$ was present at the intracellular membrane face (Demidchik et al. 2007). Overall, these results suggest spatial heterogeneity and differential sensitivity of $Ca^{2+}$ channel activation by reactive oxygen species in the root that could underpin signaling.

### *4.7.3 ROS and Programmed Cell Death*

Programmed Cell Death (PCD) plays an important role in mediating plant adaptive responses to the environment, and was experimentally proved to occur in response to salinity, cold stress, waterlogging, and hypoxia (Katsuhara and Kawasaki 1996; Pennell and Lamb 1997; Huh et al. 2002). While the regulatory mechanisms of PCD in animals are fairly well known and mostly depend on caspase activity (Lam et al. 2001), the apoptotic machinery and signal-transduction pathways of PCD in plants remain unclear. A key role for caspase-like proteases has been suggested (Hatsugai et al. 2004; Chichkova et al. 2004), and several reports of plant proteases with caspase-like properties that functionally mimic caspase activity in animals have been published (Watanabe and Lam 2004; Rojo et al. 2004).

While membrane-transport processes were shown to play a pivotal role in PCD in animal tissues (Gulbins et al. 2000; Panayiotidis et al. 2006), only few studies have been undertaken to investigate PCD-related membrane-transport processes in plants. In order to fill this gap, we investigated specific ion flux "signatures" in *Nicotiana benthamiana* plants transiently expressing CED-9 (Cell death defective-9) anti-apoptotic gene and undergoing oxidative stress. We showed that expression of CED-9 increased plant oxidative stress tolerance by altering $K^+$ and $H^+$ flux patterns across the plasma membrane (Shabala et al. 2007b). PVX (Potato virus X)/CED-9 plants were capable of preventing stress-induced $K^+$ efflux through outward-rectifying depolarization-activating $K^+$ channels (KOR) and non-selective cation channels (NSCC), so maintaining intracellular $K^+$ homeostasis. A mechanistic model for PCD

linking changes in cytosolic $K^+$ homeostasis with activation of plant proteases was suggested (Shabala 2009).

The above work, conducted on tobacco leaf mesophyll tissue, was then complemented by a comprehensive electrophysiological study on *Arabidopsis* roots (Demidchik et al. 2010). It was shown that prolonged (2–3 days) $OH^\bullet$ treatment resulted in appearance of PCD symptoms in root epidermal cells. Importantly, $OH^\bullet$- induced PCD was absent, or significantly delayed, in *gork1-1* mutants lacking functional outward-rectifying depolarization-activated GORK potassium channels (Demidchik et al. 2010). Consistent with these observations, PCD protease activation, measured by fluorescently labeled protease inhibitor zVAD-fmk (Benzyloxycarbonyl-Val-Ala-Asp (OMe)—uoromethylketone), was about three to four times lower in *gork1-1* compared with the WT. *Gork1-1* plants also revealed about four times less TUNEL (Terminal deoxynucleotidyl dUTP nick end labeling) staining after 15 h exposure to stress implying that $OH^\bullet$-activated $K^+$-efflux channels are likely to be involved in endonuclease activation caused by oxidative stress (Demidchik et al. 2010).

### 4.7.4 ROS in Stress Cross-Protection

Cross-tolerance is the synergistic co-activation of non-specific stress-responsive pathways, referring to a situation when an organism's exposure to one stress increases its tolerance to another (Mittler 2006). In most cases, induced cross-tolerance was attributed to reactive oxygen species (ROS) production during the so-called "oxidative burst"–the rapid release of $H_2O_2$—and was linked primarily with plant biotic stress responses. This oxidative burst always follows stress-induced elevation in $[Ca^{2+}]_{cyt}$ (Cessna et al. 2003) and, thus, was related to stress-induced activation of plasma membrane $Ca^{2+}$ permeable channels (Pei et al. 2000; Mori and Schroeder 2004). However, although transient increases in $[Ca^{2+}]_{cyt}$ are essential for plant responses to a variety of environmental stimuli, long-lasting elevations in $[Ca^{2+}]_{cyt}$ are harmful for cells (Bose et al. 2011b). Hence, the basal conditions must be restored back to the resting level after the signal is completed, enabling cells to react to further signals (Sanders et al. 1999; Beffagna et al. 2005). Surprisingly, the molecular identity and operating modes of active $Ca^{2+}$ efflux systems mediating this process are poorly understood.

Using *N. benthamiana* plants we have recently demonstrated that plants infected with *Potato virus X* (PVX) had a superior oxidative [UV-C (Ultraviolet-C) and $H_2O_2$] stress tolerance (Shabala et al. 2011a). These plants were able to maintain lower levels of cytosolic free $Ca^{2+}$ under stress conditions, and this effect was attributed to more active plasma membrane $Ca^{2+}$ efflux systems in PVX-inoculated plants. Pharmacological experiments coupled with biochemical and molecular assays suggested that plasma membrane $Ca^{2+}/H^+$ exchangers but not plasma membrane $Ca^{2+}$-ATPases mediate net $Ca^{2+}$ efflux under oxidative stress conditions (Shabala et al. 2011a). Also affected was $H^+$-ATPase activity, with ~40% increase in

ATP-dependent proton pumping observed in plasma membrane vesicles isolated from plants pre-treated with UV light (Shabala et al. 2011a). However, the amount of plasma membrane $H^+$-ATPase was not different between treatments, suggesting that the plasma membrane $H^+$-ATPase had been activated at the post-translational level.

So far, $Ca^{2+}/H^+$ antiporter activity has been characterized only for the tonoplast (Sanders et al. 1999; Hirschi 2001). Our data (above) provide electrophysiological evidence for the presence of such $Ca^{2+}/H^+$ exchangers also at the plasma membrane. Furthermore, using biochemical and electrophysiological approaches, we reveal that, in addition to PM $Ca^{2+}/H^+$ exchangers, both endomembrane $P_{2A}$ and $P_{2B}$ $Ca^{2+}$-ATPases play significant roles in adaptive responses to oxidative stress, and that their functional expression is significantly altered in PVX-inoculated plants (Shabala et al. 2011b). Taken together, these findings highlight the crucial role of $Ca^{2+}$ efflux systems in acquired tolerance to oxidative stress and open up prospects for practical applications in agriculture.

## 4.8 Biotic Stresses

### *4.8.1 Plant Responses to Pathogens*

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 (Nurnberger et al. 1994; Jabs et al. 1997; Blume et al. 2000; Lecourieux et al. 2002; Kadota et al. 2004). It was suggested that calcium influx is required for hypersensitive response (HR) initiation and that HR, once initiated, requires sustained $Ca^{2+}$ influx (Atkinson et al. 1990). 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 being used.

To address the above issue, we have 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* (*ultracurvata2-2/gigantea-2*) *Arabidopsis* mutant (Tegg et al. 2005).

The signaling role of $Ca^{2+}$ was further investigated in experiments using a model plant *N. benthamiana* in response to a challenge with HR-causing pathogen, *Pseudomonas syringae* (Nemchinov et al. 2008). Addition of bacterial inoculum to the measuring chamber caused a rapid and transient elevation in net $Ca^{2+}$ uptake by leaf mesophyll which peaked at $\sim 1$ min after treatment. The quickness of this response may suggest either direct association of $Ca^{2+}$ -permeable channels with plasma membrane PAMPs (Pathogen-associated molecular patterns)-recognition receptors or a very short signaling pathway from the receptors to the channels. This initial "receptor-type" $Ca^{2+}$ uptake was short-lived and disappeared within 6–10 min after the challenge (Nemchinov et al. 2008). More importantly, however, this initial calcium uptake was then followed by a well-defined calcium efflux initiated 12–48 h after the challenge. As passive $Ca^{2+}$ efflux from the cell cytosol is thermodynamically impossible, some active $Ca^{2+}$ efflux system must be involved. Given $Ca^{2+}$ flux sensitivity to cyclopiazonic acid (a known inhibitor of $Ca^{2+}$-ATPase), $Ca^{2+}$-ATP pump involvement was suggested (Nemchinov et al. 2008).

In the light of the above, calcium signaling in response to pathogens is most likely a multi-step process and consists of several phases. It appears that calcium acts not only as an important second messenger in the activation of resistance responses (Grant et al. 2000, Balagué et al. 2003) but also as a downstream mediator of later cell death acceleration, inhibition of the spread of invading pathogens and completion of defense reaction. Accordingly, it was suggested that the existing model of HR should be amended to include such $Ca^{2+}$ pumps (Nemchinov et al. 2008).

### *4.8.2 Plant Responses to Viral Infection*

While the role of membrane-transport processes in plant-pathogen interaction is well defined (see above), electrophysiological events mediating plant responses to viral infection are essentially unexplored. In a rare report on non-host hypersensitive response (HR) to papaya mosaic virus (PMV), Schvarzstein (1997)

described a decrease in the average inward currents and an increase in the outward currents from protoplasts isolated from *Gomphrena globosa* leaf tissue, using the patch-clamp technique. It was suggested that several ions such as $K^+$, $Cl^-$, gluconate, and $Ca^{2+}$ may contribute to the above currents, and that cell membrane damage is required for viral infection (Schvarzstein 1997).

In our recently published work, we used the MIFE technique to measure net ion fluxes from mesophyll tissue from a range of host and non-host plants in response to infection with *Potato virus X*, PVX (Shabala et al. 2010a). Addition of the purified PVX preparation to the tobacco mesophyll tissue caused no changes in the rate of $Ca^{2+}$ transport across the plasma membrane. Also, no significant changes in $[Ca^{2+}]_{cyt}$ were detected for at least 50 min after PVX treatment suggesting that $Ca^{2+}$ release from internal stores was also not a part of the signal-transduction mechanism in plant-viral interaction. Thus, it appears that, contrary to bacterial pathogens, rapid $Ca^{2+}$ signaling may not be essential for the viral perception and initiation of downstream transduction pathway. Instead, a massive $K^+$ efflux was measured as early as 10 min after PVX inoculation (Shabala et al. 2010a). This efflux was absent in non-host species, suggesting high host-specificity of the process. This may suggest that viral infections trigger ionic currents associated with plant defense signaling that differ from ion fluxes induced by other microbes.

Pharmacological and membrane potential data in our experiments point out that a significant part of measured $K^+$ flux was mediated by depolarization-activated outward-rectifying $K^+$ channels. Recently, the phenomenon of rapid $K^+$ release from host cells during the early phase of viral infection was reported for *Chlorella* cells infected by PBCV-1 (*Paramecium bursaria* chlorella virus) (Neupartl et al. 2008); this phenomenon was explained by incorporation of viral-encoded $K^+$ channels (Kcv) into the host membrane. It remains to be answered whether the same scenario is also applicable for higher plants.

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