Effect of divalent cations on ion fluxes and leaf photochemistry in salinized barley leaves

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Abstract
Photosynthetic characteristics, leaf ionic content, and net fluxes of Na+, K+, and Cl− were studied in barley (Hordeum vulgare L) plants grown hydroponically at various Na/Ca ratios. Five weeks of moderate (50 mM) or high (100 mM) NaCl stress caused a significant decline in chlorophyll content, chlorophyll fluorescence characteristics, and stomatal conductance (gs) in plant leaves grown at low calcium level. Supplemental Ca2+ enabled normal photochemical efficiency of PSII (Fv/Fm around 0.83), restored chlorophyll content to 80–90% of control, but had a much smaller (50% of control) effect on gs. In experiments on excised leaves, not only Ca2+, but also other divalent cations (in particular, Ba2+ and Mg2+), significantly ameliorated the otherwise toxic effect of NaCl on leaf photochemistry, thus attributing potential targets for such amelioration to leaf tissues. To study the underlying ionic mechanisms of this process, the MIFE technique was used to measure the kinetics of net Na+, K+, and Cl− fluxes from salinized barley leaf mesophyll in response to physiological concentrations of Ca2+, Ba2+, Mg2+, and Zn2+. Addition of 20 mM Na+ as NaCl or Na2SO4 to the bath caused significant uptake of Na+ and efflux of K+. These effects were reversed by adding 1 mM divalent cations to the bath solution, with the relative efficiency Ba2+ > Zn2+ = Ca2+ > Mg2+. Effect of divalent cations on Na+ efflux was transient, while their application caused a prolonged shift towards K+ uptake. This suggests that, in addition to their known ability to block non-selective cation channels (NSCC) responsible for Na+ entry, divalent cations also control the activity or gating properties of K+ transporters at the mesophyll cell plasma membrane, thereby assisting in maintaining the high K/Na ratio required for optimal leaf photosynthesis.

Key words: Barley, chlorophyll fluorescence, divalent cations, ionic fluxes, leaf photochemistry, photosynthesis, stomatal conductance.

Introduction
The detrimental effects of salinity can be partially alleviated by external Ca2+ (LaHaye and Epstein, 1969; Cramer et al., 1989; Martinez and Lauchli, 1993; Reid and Smith, 2000; Cramer, 2002; Shabala et al., 2003). Recent studies provided some evidence that not only Ca2+ 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). A patch-clamp study on Arabidopsis roots showed that non-selective cation channels, NSCC (a major route for Na+ entry into the cell; Maathuis and Amtmann, 1999; Tyerman and Skerret, 1999), were efficiently blocked, and not only by Ca2+ but also by Ba2+ and Zn2+ (Demidchik and Tester, 2002). Davenport and Tester (2000) also showed a direct effect of extracellular Mg2+ on NSCC activity. Is the beneficial role of divalent cations in plant adaptive responses to salinity limited to the control of Na+ transport across the plasma membrane in saline conditions? This issue has never been adequately addressed in the literature.

It has previously been reported (Shabala et al., 2003) that supplemental Ca2+ significantly ameliorated the detrimental effects of salinity on the growth and root nutrient acquisition of hydroponically grown barley plants. It was of particular interest that the shoot length of plants grown in a saline environment (100 mM NaCl) at a high (10 mM) Ca2+ level was not significantly different from control plants (grown at 1 mM NaCl). At the same time, the root length of those plants grown at 100 mM NaCl with 10 mM Ca2+...
Ca\(^{2+}\) was only 30\% of the control value. Earlier experiments on salt-treated broad bean mesophyll tissues (Shabala, 2000) showed that additional Ca\(^{2+}\) in the bathing solution prevents the leak of K\(^{+}\) from those tissues, an effect similar to that observed in salinized barley roots (Shabala et al., 2003). However, it was not clear whether the ameliorative effect of Ca\(^{2+}\) on shoot growth was merely a consequence of better root performance, or whether there is a specific effect of Ca\(^{2+}\) on the physiological processes in the leaf tissues, in particular on photosynthesis.

The effect of salinity on photosynthesis is complex. Elevated, albeit low, salinity levels sometimes enhance photosynthetic performance (Greenway and Munns, 1980). At medium or high salinity, leaf photosynthesis is severely inhibited (Seemann and Critchley, 1985; Bethke and Drew, 1992). Substantial reduction in net CO\(_2\) assimilation under saline conditions was reported for many species including barley (Belkhodja et al., 1999; Toker et al., 1999). It was also shown that supplemental Ca\(^{2+}\) may partially restore the CO\(_2\) assimilation rate (Huang and Redman, 1995). The underlying mechanisms are poorly understood, and it remains to be shown whether the same effect is attributable to other divalent cations as well.

One possible reason for the observed salt-induced decrease in photosynthetic rates could be a reduction in chlorophyll content (usually associated with leaf chlorosis). Many papers have reported a salt-induced decrease in the total amount of chlorophyll in affected leaves, as well as changes in the chlorophyll \(a:b\) ratio (Singh and Dubey, 1995; Delfine et al., 1999). Apart from causing ion toxicity and disrupting cell metabolism, salinity may also affect plant photosynthetic performance indirectly by reducing stomatal conductance \(g_s\) via the osmotic component of salt stress (Munns, 2002). A significant decrease in \(g_s\) has always been considered an important component of the overall reduction in net CO\(_2\) assimilation in affected plants (Delfine et al., 1999; Belkhodja et al., 1999).

It is unclear which of the above components (stomatal and non-stomatal) of plant photosynthetic performance may be affected by supplemental calcium, and to what extent such amelioration may occur. It also remains to be answered whether this effect is Ca\(^{2+}\)-specific, and whether the application of divalent cations may also have a beneficial impact on leaf photochemistry. Last but not least, underlying ionic mechanisms of such amelioration remain to be elucidated. Some of these issues are addressed in this paper.

Materials and methods

Plant material and growth conditions

For whole-plant experiments, barley (Hordeum vulgare cv. Franklin) plants were grown from seeds hydroponically in modified half-strength Hoagland solution with different Na/Ca ratios as described by Shabala et al. (2003). Altogether, three different levels of Ca\(^{2+}\) (0.1 mM, low; 1.0 mM, intermediate, and 10 mM, high) and three different levels of Na\(^{+}\) (1, 50, and 100 mM) were used in a full factorial experiment. The choice of these Ca\(^{2+}\) concentrations was determined by (i) an attempt to make the results comparable with other literature data (Davenport et al., 1997) and (ii) the fact that a half-maximal block of NSCC by external Ca\(^{2+}\) is at around \(A_{Ca}=0.1\) mM (Demidchik and Tester, 2002). Therefore, in the experimental conditions used here (low Ca treatment, \(A_{Ca}=0.036\) mM), less than 10\% of NSCC was blocked under saline conditions. The whole plant experiment was performed three times in 2001–2003, twice at the University of Tasmania, Hobart, Australia and once at the University of Washington, Seattle, USA, with similar results.

For ion flux measurements and experiments on excised leaves, plants were grown from seeds in fertilized potting mix in 0.5 l plastic pots essentially as described by Shabala (2000). The plants were grown under 16/8 h light/dark regime (model M1500-A lighting unit; Thorne, Moonah, Australia; total irradiance 150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) at the leaf level) at ambient temperature 20 \(^{\circ}\)C (dark) and 26 \(^{\circ}\)C (light). Leaves from 3–4-week-old plants were used for measurements.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence emission from the upper surface of the leaves was measured at room temperature using a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). All measurements were carried out in the saturation pulse method described in the Mini-PAM manual. This portable fluorometer measures key parameters of chlorophyll fluorescence induction kinetics such as \(F_o\), \(F_m\), \(F_{in}\), and \(F_v/F_o\) for both light- and dark-adapted samples. All measurements were performed on the youngest fully expanded leaf, approximately one-third of the leaf length away from the tip. At least 20 min were allowed for leaves to adapt to light conditions. For light-adapted measurements, an external light source was used, providing background irradiation of about 90 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). \(F_m\) was induced by an 800 ms pulse of intense white light (quantum intensity of the source up to 10 000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) ). The variable to maximum fluorescence ratio was then calculated as \(F_m/F_{in}=\left(F_{in}-F_o\right)/F_{in}\). More specific details on chlorophyll fluorescence measurements can be found in previous publications (Shabala et al., 1998; Smethurst and Shabala, 2003).

Pigment analysis

About 100 mg of the leaf were taken from the upper part of the youngest fully expanded leaf using a sample punch. The sample was put into a 10 ml screw-top vial, and 5 ml of 96\% methanol was added, as well as a few crystals of MgCO\(_3\) (to neutralize released organic acids). Samples were kept in a refrigerator at + 4 \(^{\circ}\)C, until all the chlorophyll was extracted and the leaf discs became colourless. Then 3 ml aliquots of each sample were taken, and the optical densities at two different wavelengths, 665 and 649 nm, were measured using a UV-Visible Spectrophotometer (Beckman DU-40). The amounts of chlorophyll \(a\) and \(b\) in the extract were calculated using the following equations (Smethurst and Shabala, 2003),

\[
C_a (\text{mg} \, l^{-1}) = 13.70 D_{665} - 5.76 D_{649} \\
C_b (\text{mg} \, l^{-1}) = 25.80 D_{649} - 7.60 D_{665}
\]

and the results were expressed in mg Chl \(l^{-1}\) leaf FW.

Transpiration and stomatal conductance

Transpiration rate and stomatal conductance to H\(_2\)O vapour were measured using a Delta-T MK3 porometer (Delta-T devices, Cambridge, UK). Measurements were taken on a sunny day from
the mid-lamina portion of the abaxial surface of the youngest fully expanded leaf 1 d before harvesting.

Elemental content in leaves

About 0.3 g dry and ground plant material was placed in a digestion tube and 2.5 ml of the digestion mixture (H₂SO₄–Se–salicylic acid) was added. After mixing, the tube was allowed to stand for 2 h and was then placed into a heating block and heated for 2 h at 100 °C. After cooling, three 1 ml aliquots of H₂O₂ were added. After each addition, the contents of the tube were thoroughly mixed. Then the tube was placed in an aluminium block and heated to 330 °C (just below the boiling point of the digestion mixture). The digestion was complete in about 2 h.

The cooled, clear digest was diluted to 20 ml with distilled water, filtered, and aliquots were taken for analyses. Potassium and sodium content of these plant samples was measured using an EEL flame photometer (Evans Electroselenium Ltd, Halstead, England). Leaf Ca content was analysed using atomic absorption spectroscopy (Varian, Melbourne, Australia). Each sample was run twice, and each variant had 4–6 replicates analysed.

Net ion flux measurements

Net fluxes of Na⁺, K⁺, and Cl⁻ were measured from isolated leaf mesophyll using the MIFE non-invasive ion flux measuring micro-electrode technique essentially as described in previous publications (Shabala, 2000; Shabala and Shabala, 2002; Shabala et al., 2003). Briefly, microelectrodes were pulled from borosilicate glass capillaries, oven-dried, and silanized with tributylchlorosilane. Dried and cooled electrode blanks were then back-filled with appropriate back-filling solutions (0.5 M NaCl solution for Na⁺ and Cl⁻; 0.2 M KCl for K⁺), and the electrode tip front filled with commercially available ionophore cocktails (catalogue number 24902 for Cl⁻: 60031 for K⁺, and 71176 for Na⁺; all from Sigma-Aldrich). The electrodes were calibrated in sets of standard solutions (0.1–50 mM) before and after use. Electrodes with a response of less than 50 mV per decade and correlation less than 0.999 were discarded.

Four to five hours prior to the experiment, the youngest fully expanded leaf was excised and the abaxial epidermis was removed from a small area by fine forceps. Then small leaf segments (c. 5×8 mm) were cut and floated (exposed mesophyll down) in aerated experimental solution essentially as described by Shabala (2000).

Approximately 4 h after removing the epidermis, a suitable segment was mounted in a Perspex holder and placed on a three-dimensional hydraulic manipulator, as described by Shabala (2000). Specific details on the ionic composition of the experimental solution and the duration of the treatment are given in the text below. The microelectrode tips were put close together and positioned 50 µm above the peeled leaf surface (mesophyll) during ion flux measurements. The travel range of the ion-selective probes was from 50–90 µm, and the time at each position was 5 s. Steady-state ion fluxes were measured for 10–20 min. Then the treatment was given, and measurements of the transient ion flux kinetics were taken for another 40–50 min. Two major types of MIFE experiments were conducted. One protocol included incubation of the mesophyll segment in basic salt medium, BSM (0.1 mM CaCl₂+0.2 mM KCl+0.5 mM NaCl) and measuring ion flux kinetics in response to 20 mM Na⁺ salt stress (given as either 20 mM NaCl or 10 mM Na₂SO₄). This concentration was chosen based on the expected level of Na⁺ in the leaf apoplast (Muhling and Lauchli, 2002). The second protocol involved the incubation of the leaf segment for 2–3 h in 20 mM Na⁺ added to the BSM solution, and measuring net fluxes of K⁺, Na⁺, and Cl⁻ in response to 1 mM of diivalent cation (chloride salts of Ca²⁺, Mg²⁺, Ba²⁺, or Zn²⁺) added to the bath. To eliminate a confounding effect of additional Cl⁻ added to the bath during Cl⁻ flux measurements, Na₂SO₄ was used instead of NaCl in these experiments.

Calcium content in the sap extract

Leaf samples were excised, wrapped in aluminium foil and immediately frozen using dry ice. Samples were sealed in a double layer of ziplock bags and transferred to a −80 °C cryo freezer (REVCO, Rheem Manufacturing, Asheville NC). For measurements, samples were thawed and then centrifuged at 10 000 rpm for 6 min to release the bulk sap extract. Calcium concentration in the bulk leaf sap extract was measured using a MIFE ion-selective Ca microelectrode from a 100 µl aliquot of each sample. Specific details on Ca²⁺ microelectrode fabrication and calibration were essentially as described by Shabala (2000).

Results

Salinity stress caused a dramatic reduction of chlorophyll content in leaves of barley plants grown at low Ca²⁺ level in the hydroponic solution (Fig. 1). Five weeks growth at 100 mM NaCl and 0.1 mM Ca²⁺ resulted in about a 4-fold decrease in total chlorophyll content compared with the control (1 mM NaCl; Fig. 1A). The effect was more severe on chlorophyll a and, as a result, the Chl a/b ratio declined significantly (Fig. 1B).
Growing plants with supplemental Ca\(^{2+}\) largely prevented the detrimental impact of salinity on chlorophyll content (80–90% of 1 mM Na\(^+\)). No significant difference between 1 mM and 10 mM Ca\(^{2+}\) was found. There was no significant difference in either Chl \(a\) or \(b\) content between 1 mM NaCl and 50 mM NaCl treatments when supplemental Ca\(^{2+}\) was present during growth (Fig. 1).

Severe NaCl-induced inhibition of the photochemical efficiency of PSII was found when parameters of chlorophyll fluorescence were measured (Figs 2, 3). In dark-adapted samples, \(F_v/F_m\) values drop dramatically from about 0.83 in controls (an indication of healthy plants) to as low as 0.35 (high Na\(^+\) treatment; Fig. 2A) in salt-stressed leaves. Supplemental Ca\(^{2+}\) completely prevented the detrimental effects of salinity on photochemical efficiency of PSII (all \(F_v/F_m\) values are above 0.8; Fig. 2A). Similar trends were found when light-adapted samples were measured (Fig. 3).

Stomatal conductance (\(g_s\)), measured after the 5 weeks of salt stress, was dramatically reduced by a high Na/low Ca combination (Fig. 4). Supplemental Ca\(^{2+}\) significantly (\(P < 0.05\)) ameliorated the detrimental effect of salinity on \(g_s\). However, the recovery was not complete, and even at 10 mM Ca\(^{2+}\), \(g_s\) values for 100 mM NaCl were about 50% of controls (Fig. 4).

Leaf nutrient analysis showed a significant (\(P < 0.01\)) increase in Na\(^+\) content and a decrease in K\(^+\) content in leaves of plants grown at high Na/low Ca treatments (Table 1). Growth of plants with supplemental Ca\(^{2+}\) significantly reduced the amount of Na\(^+\) in leaf tissues, the effect of 10 mM Ca\(^{2+}\) being stronger than 1 mM Ca\(^{2+}\). Supplemental Ca\(^{2+}\) also significantly ameliorated the salinity-induced decrease of leaf total K\(^+\) (Table 1). In general, the ameliorative effect of supplemental Ca\(^{2+}\) on leaf K\(^+\) was at least as strong as on Na\(^+\), if not stronger (Table 2).

Both overall Ca\(^{2+}\) content (expressed as % DW; Table 1) and leaf Ca\(^{2+}\) content (measured in the bulk leaf sap extract; Table 3) decreased with increasing salinity (with the only exception being at very low Ca) and significantly (\(P=0.01\)) increased with increased Ca availability to plants. Ameliorative effects of Ca\(^{2+}\) were also observed on excised leaves (Fig. 5). The youngest fully expanded leaves from 4-week-old plants grown in fertilized potting mix were excised and the cut ends of each leaf base immersed in a BSM solution containing 50 mM NaCl under laboratory conditions. After 2 d of treatment, a significant (\(P < 0.01\)) decrease in the maximum efficiency of PSII (judged by the \(F_v/F_m\) value) was observed (Fig. 5). This detrimental effect of NaCl on leaf photochemistry was much less when 1 mM of a divalent cation (Ca\(^{2+}\), Mg\(^{2+}\), or Ba\(^{2+}\)) had been included in the medium (\(P < 0.01\); Fig. 5). It should be noted that overall \(F_v/F_m\) values of excised leaves were slightly lower than those measured from intact plants, even
in the controls, probably reflecting the overall inhibition of photosynthetic activity in excised leaves.

To provide some insights into the ionic mechanisms of the above amelioration of the detrimental effects of NaCl on leaf photochemistry, net ion fluxes of Na⁺, K⁺, and Cl⁻ were measured from leaf mesophyll tissue in response to salt stress. To make the results comparable with in planta processes under natural conditions, a relatively low (20 mM Na⁺) salt concentration was chosen. This concentration was the one typically found in the apoplast of salinized leaves of most species (Muhling and Lauchli, 2002).

Immediately upon salt treatment (20 mM NaCl or 10 mM Na₂SO₄), a large Na⁺ influx was observed (Fig. 6A). At the end of the measurements (30 min after NaCl application), a small influx of Na⁺ (c. 10% of the initial value) was still present, although due to rather large noise, originating from the low sodium flux sensitivity in the presence of high Na⁺, these values were not very different from zero.

Chloride influx in response to Na⁺ (Na₂SO₄) treatment was much smaller than Na⁺ influx, and returned back to control levels about 15 min after treatment was applied (Fig. 6B). In contrast to the monophasic transient Na⁺ and...
Cl⁻ flux responses, K⁺ response had two clearly pronounced phases. The first phase was characterized by a significant ($P < 0.01$) K⁺ efflux measured immediately after NaCl application. This efflux gradually wound down, with K⁺ flux reaching control levels 15–20 min after the treatment began. During the second phase (from 20 min after treatment) a gradual shift towards a higher K⁺ efflux was observed (Fig. 6C). Significantly ($P < 0.01$) higher K⁺ efflux was measured from salinized leaf mesophyll several hours after the treatment (Fig. 6C, inset). The presence of 20 mM TEACl in the bath completely prevented Na⁺-induced K⁺ efflux from leaf mesophyll (open symbols in Fig. 6C).

Most of the observed effects were reversed when physiological (1 mM) concentrations of divalent cations were added to leaf mesophyll segments pretreated for several hours in BSM solution containing 20 mM Na⁺ (Figs 7–9). Transient sodium efflux was measured immediately following treatment (Fig. 7). The effect was only transient, and 30 min after treatment, Na⁺ fluxes had returned to their original values of about 200 nmol m⁻² s⁻¹ for all ions except Ba²⁺.

By contrast, the effect of divalent cations on the net K⁺ flux was more prolonged. NaCl-induced K⁺ efflux was significantly reduced by the application of 1 mM of all divalent cations (chloride salts), with Ba²⁺ once again
being the most effective (net shift towards influx of about 120 nmol m\(^{-2}\) s\(^{-1}\)), followed by Ca\(^{2+}\) and Zn\(^{2+}\) (50–60 nmol m\(^{-2}\) s\(^{-1}\)) and then Mg\(^{2+}\) (40 nmol m\(^{-2}\) s\(^{-1}\)) (as judged by the difference in steady-state values before and after treatment; Fig. 8).

Finally, the addition of the divalent cations caused a significant and prolonged increase in net Cl\(^{-}\) uptake (Fig. 9). Again, the most effective was Ba\(^{2+}\), followed by other cations. At the end of the treatment, significant (P <0.05) net influx of Cl\(^{-}\) was measured for all treatments (Fig. 9).

**Discussion**

**Leaf photochemistry as a major beneficiary**

Supplemental Ca\(^{2+}\) significantly prevented detrimental effects of salinity on leaf pigment composition (Fig. 1), stomatal conductance (Fig. 4), and leaf photochemistry (Figs 2, 3) in hydroponically grown plants. The extent of this effect was, however, different for ‘stomatal’ and ‘non-stomatal’ components of photosynthesis. Despite significant improvement (high Ca/high Na treatments versus low Ca/high Na treatments), stomatal conductance (g\(_{s}\)) values were still significantly (P <0.05) lower (c. 50%) than control (low Ca/low Na plants). At the same time, an almost complete recovery of leaf photochemistry was observed (Figs 2, 3).

Stomatal limitation of photosynthesis arises from decreased g\(_{s}\) in response to salt stress (Cramer *et al.*, 1989; Wright *et al.*, 1993; Rivelli *et al.*, 2002). Munns (2002) argues that the g\(_{s}\) decrease is likely to be caused by the osmotic component of salt stress. As a result of reduced g\(_{s}\), both transpiration and CO\(_{2}\) assimilation rates are severely reduced; less water becomes available for extension growth in shoot tissues, and fewer carbohydrates are produced, eventually affecting both root and shoot growth (Rivelli *et al.*, 2002).

There are only a few, controversial, reports on the effects of supplemental Ca\(^{2+}\) on stomatal functioning in response to salinity stress. Perera *et al.* (1995) reported that elevated Ca\(^{2+}\) levels reduced detrimental NaCl effects on g\(_{s}\) in both intact cotton plants and isolated epidermis. However, additional Ca\(^{2+}\) was not able to ameliorate NaCl-induced reduction in g\(_{s}\), transpiration, and xylem water potential in blueberry (Wright *et al.*, 1993). In these experiments, at high Na/high Ca ratios, stomatal conductance of barley leaves was only at 50% of control levels (Fig. 4). Therefore, under the conditions used here it appears that, although there is some beneficial effect of supplemental Ca\(^{2+}\) on g\(_{s}\), the effect is indirect and its contribution towards improved plant growth under saline conditions may not be great.

Although there is some scepticism in the literature over whether chlorophyll degradation is the primary cause of photosynthetic degeneration and, therefore, a main biochemical factor of the observed growth reduction (Everard *et al.*, 1994), the NaCl-induced decrease in the chlorophyll level is widely reported (Abdullah *et al.*, 2001; Kaya *et al.*, 2001; Renault *et al.*, 2001). A several fold decrease in leaf chlorophyll content was also found in salt-stressed leaves grown at low Ca\(^{2+}\) levels (Fig. 1A). Supplemental Ca\(^{2+}\) almost completely recovered chlorophyll levels (Fig. 1), consistent with other reports (Ebert *et al.*, 2002). Supplemental Ca\(^{2+}\) also almost completely prevented the loss of photochemical efficiency of PSII in both dark-adapted (Fig. 2) and light-adapted (Fig. 3) leaves. The F\(_{v}/F\(_{m}\) values for all barley leaves were around 0.83, even at 100 mM NaCl, when receiving supplemental Ca\(^{2+}\) (Fig. 2A). This is an indication of a ‘very healthy’ plant, with its photochemistry operating optimally. As far as the authors know, there are no previous reports on the effects of supplemental Ca\(^{2+}\) on chlorophyll fluorescence parameters in salt-stressed plants.

Taken together, the results suggest that the ameliorative effect of supplemental Ca\(^{2+}\) on photosynthesis was achieved primarily via biochemical pathways rather than through the regulation of stomatal conductance and CO\(_{2}\) availability to plants, although the ‘stomatal’ component also benefited from supplemental Ca\(^{2+}\).

**Specific targets for supplemental Ca\(^{2+}\) are present in leaves as well as roots**

The large number of factors potentially contributing to the observed effects always complicates the interpretation of whole-plant data.

It has almost been taken for granted that an ability of plants to minimize net Na\(^{+}\) uptake by roots is the main
feature conferring salt tolerance (Munns, 2002). That could be achieved either by restriction of Na+ uptake into the root epidermis (Maathuis and Amtmann, 1999), or by enhanced activity of a plasma membrane H+/Na+ exchanger, contributing to Na+ removal from the root (Tester and Davenport, 2003). Each of these is a potential target for the ameliorative effect of supplemental Ca2+. Therefore, it might be argued that the beneficial effects of supplemental Ca2+ on leaf photochemistry could merely be due to a restriction of Na+ uptake by plant roots or by preventing the excessive Na+ from being delivered to the shoot. Indeed, Table 1 shows the reduced Na+ content in leaves grown with supplemental Ca2+.

Another issue arises from the fact that low Ca (0.1 mM) solution was used to grow control plants. Being justified by the requirements that activity of NSCC is not inhibited (see Materials and methods and Demidchik and Tester, 2002, for details), this concentration is somewhat lower than in most typical soils or than is usually used for hydroponic plant growth (Marschner, 1995). Under high salinity conditions (100 mM NaCl) the actual Ca2+ activity will be only 36 μM (Shabala et al., 2003). Thus, it can be argued that basic Ca2+ deficiency may be a reason for the substantial decline in root growth and, consequently, poor plant performance at 50 or 100 mM NaCl.

An important argument rebutting these concerns comes from experiments on excised leaves. Ameliorative effects were also observed when Ca2+ was added directly to excised leaves – into xylem flow (Fig. 5). After 2 d of treatment in 50 mM NaCl, a significant (P < 0.01) decrease in the maximum efficiency of PSII (Fv/Fm as low as 0.58) was observed (Fig. 5). This effect was significantly prevented when 1 mM Ca2+ was present in the treatment solution (Fv/Fm values recovered to ~0.7). The fact that such amelioration was also observed in response to other divalent cations such as Mg2+ and Ba2+ (Fig. 5), strongly suggests that the observed effect is not merely a result of overcoming plant Ca2+ deficiency under saline conditions.

Taken together, the data suggest that, although supplemental Ca2+ indeed restricts net Na+ uptake by barley roots under saline conditions and reduces Na+ accumulation in the shoot (Table 1), it is not likely that Na+ transporters in epidermal root cells represent the only target for ameliorative Ca2+ effects on leaf photochemistry. It appears that there is at least one more additional mechanism present, namely direct control of ion uptake into leaf mesophyll by elevated levels of Ca in the leaf (Table 3).

Regulation of K transport is central to amelioration of salinity by divalent cations

In addition to reduced Na+ content in leaf tissues, the beneficial effects of supplemental Ca2+ on leaf photosynthesis may be a result of much improved K+/Na+ ratios in leaf mesophyll cells due to the regulation of the leaf K+ balance. An ability of plants to retain K+ and to maintain K+/Na+ selectivity has always been considered a key feature of salt tolerance (Maathuis and Amtmann, 1999; Munns, 2002; Tester and Davenport, 2003). In the experiments, nutrient analysis of leaf tissues showed that supplemental Ca2+ affected both Na+ and K+ content, with ameliorative effects on leaf K+ being at least as strong as on Na+ (Table 1).

Reid and Smith (2000) hypothesized that increasing Ca2+ concentrations may restore the selectivity of potassium channels. Zhong and Lauchli (1994) concluded that one possible mechanism by which supplemental Ca2+ alleviates the inhibitory effects of NaCl on cotton root growth is by maintaining plasma membrane selectivity of K+ over Na+. A wheat mutant with enhanced capacity for K+ accumulation in leaves was more salt-tolerant than a wild type (Rascio et al., 2001). The recently described salt-tolerant stt2 mutation in the fern Ceratopteris richardii involves an enhanced influx of K+ and higher selectivity for K+ over Na+ (Warne et al., 1995). Thus, it appears that K+ transporters may be another target for supplemental Ca2+ in mediating ameliorating Ca2+ effects in salinized plants, in addition to the regulation of NSCC (Demidchik and Tester, 2002; Tester and Davenport, 2003).

The above view is strongly supported by the ion flux data (Figs 7, 8). Not only Ca2+, but also other divalent cations (e.g. Ba2+, Mg2+, and Zn2+) significantly affected Na+ transport across the plasma membrane of salinized leaves (Fig. 7). However, divalent cation-induced Na+ efflux was very short-lived, with net Na+ fluxes stabilizing at the initial (steady-state) level 10–15 min after treatment was given (Fig. 7). Taking Ca2+ as an example and assuming an average Na+ efflux of 110 nmol m−2 s−1 over the first 10 min after Ca2+ application (Fig. 7), for a typical mesophyll cell of 25 μm diameter, about 5×10−14 moles of Na+ will be exported from the cytosol across the plasma membrane through the cell surface in response to the application of additional Ca2+. Although the precise levels of cytosolic Na+ in salinized leaf cells are not known, estimates based on X-ray analysis or the application of microelectrode techniques suggest that, in most cell types, cytosolic Na+ is around 100 mM in saline conditions (Binzel et al., 1988; Maathuis and Amtmann, 1999), with some authors reporting even higher (180–240 mM) values (Flowers and Hajibagheri, 2001). Therefore even working at the low end of this scale (i.e. assuming cytosolic free Na+ equals 50 mM), for a typical mesophyll cell of 50 μm diameter, and cytosolic volume of about 10%, the total amount of Na+ in the cytosol will be 3.2×10−13 moles. Hence, the observed Na+ efflux in response to an additional 1 mM Ca2+ treatment may contribute at most a 15% reduction of overall Na+ in the leaf cell cytosol. If the Na+ content in the cytosol is higher than 50 mM, the effect will be even smaller. Moreover, a part of the observed transient Na+ efflux may be due to Ca2+/Na+ exchanges in the Donnan
system of the cell wall (Ryan et al., 1992), further reducing the above 15% estimate. Therefore, it is unlikely that such short-lived Na\(^+\) efflux in response to additional Ca\(^{2+}\) (or other divalent cations) is solely responsible for the observed beneficial effects on leaf photochemistry either in experiments on excised leaves (Fig. 5), or in the hydroponics experiments (Figs 2, 3).

It is far more likely that the observed beneficial effects of divalent cations are due to their exerting control over the activity of plasma membrane K\(^+\) transporters. Significantly higher (P < 0.01) K\(^+\) efflux was observed after 4 h of mesophyll exposure to 20 mM NaCl (Fig. 6C, inset). All divalent cations tested caused a significant and prolonged shift towards net K\(^+\) influx (Fig. 8), with steady-state K\(^+\) flux values at the end of measurement being significantly more positive than before treatment. The presence of TEA in the bath solution completely inhibited NaCl-induced K\(^+\) efflux (Fig. 6C), so K\(^+\) flux responses could not be attributed to Donnan exchange in the cell wall. Together with beneficial effects on Na\(^+\) transport into salinized leaf tissue, this prolonged shift towards higher K\(^+\) influx will significantly improve the cytosolic K/Na ratio and, thus, provide the optimal ionic environment for leaf photochemistry.

Can chloride act as a charge-balancing ion?

It was shown previously that one of the beneficial Ca\(^{2+}\) effects on ionic relations in plant roots is the restoration of (otherwise depolarized) membrane potential (Shabala et al., 2003). This can be achieved either by increasing cation efflux or by enhanced uptake of anions across the plasma membrane. Although Na\(^+\) efflux induced by Ca\(^{2+}\) (and other divalent cations) could fit that requirement, the effect was only short-lived (15 min on average; Fig. 7). At the same time, a prolonged influx of K\(^+\) is observed (Fig. 8) which would lead to further membrane depolarization. This influx also must be electrically balanced by some other charge transfer across the plasma membrane. From these data, Cl\(^-\) may be a suitable candidate for this role. Analysis of divalent cation-induced net K\(^+\) and Cl\(^-\) fluxes (Figs 7 and 8, respectively) shows that, for all treatments, the observed K\(^+\)/Cl\(^-\) stoichiometry between ion flux changes is roughly 1:1. This is also consistent with Fernandez-Ballester et al. (1997) who showed that uptake of Cl\(^-\) was enhanced and maintained at higher than control levels in salt-stressed Phaseolus plants when 5 mM Ca\(^{2+}\) was added to the growth solution. Therefore, enhanced Cl\(^-\) uptake may contribute to charge balance, when supplemental Ca\(^{2+}\) mediates K\(^+\) influx (as needed for optimal leaf photochemistry). The Cl\(^-\) uptake is thermodynamically ‘uphill’ and is likely to be mediated by a H\(^+\)/Cl\(^-\) or 2H\(^+\)/Cl\(^-\) symporter. This would provide for the Cl\(^-\) influx, while the H\(^+\) extruding ATPase, to maintain cytosolic pH, would remove the symported H\(^+\) and provide the repolarization.

Conclusions

It appears that amelioration of salinity by supplemental Ca\(^{2+}\) is not limited to a single ion transport system or to a specific location in plants. From the above results, it is suggested that one of the ameliorative mechanisms by which leaf photochemistry benefits from supplemental Ca\(^{2+}\) is in maintaining an optimal K/Na ratio in the cytosol by regulating K\(^+\) transport across the plasma membrane of mesophyll cells. It also appears that this regulation is not specific to Ca\(^{2+}\) but can also be performed by other divalent cations including Mg\(^{2+}\), Ba\(^{2+}\), and Zn\(^{2+}\). Further studies are needed to answer the question as to which specific K\(^+\) transport system is targeted.

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