

Sequential depolarization of root cortical and stelar cells induced by an acute salt shock – implications for Na⁺ and K⁺ transport into xylem vessels

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ABSTRACT

Early events in NaCl-induced root ion and water transport were investigated in maize (*Zea mays* L) roots using a range of microelectrode and imaging techniques. Addition of 100 mM NaCl to the bath resulted in an exponential drop in root xylem pressure, rapid depolarization of trans-root potential and a transient drop in xylem K⁺ activity (A_{K^+}) within ~1 min after stress onset. At this time, no detectable amounts of Na⁺ were released into the xylem vessels. The observed drop in A_{K^+} was unexpected, given the fact that application of the physiologically relevant concentrations of Na⁺ to isolated stele has caused rapid plasma membrane depolarization and a subsequent K⁺ efflux from the stelar tissues. This controversy was explained by the difference in kinetics of NaCl-induced depolarization between cortical and stelar cells. As root cortical cells are first to be depolarized and lose K⁺ to the environment, this is associated with some K⁺ shift from the stelar symplast to the cortex, resulting in K⁺ being transiently removed from the xylem. Once Na⁺ is loaded into the xylem (between 1 and 5 min of root exposure to NaCl), stelar cells become more depolarized, and a gradual recovery in A_{K^+} occurs.

Key-words: barley; maize; membrane potential; trans-root potential; xylem loading; xylem pressure.

Abbreviations: A_{K^+} , potassium activity in the xylem; MP, membrane potential; P_x , xylem pressure; TRP, trans-root potential; A/D, analogue to digital; EDX, energy dispersive X-ray analysis; NMG, N-methyl-D-glucamine; Mes, 2-(N-morpholino) ethanesulphonic Acid; NSCC, nonspecific cation channel.

INTRODUCTION

When a root is challenged with a sudden salt shock, this treatment has a dramatic impact on ion fluxes at the root

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surface as well as on the MP of cortical cells. NaCl-induced K⁺ efflux and membrane depolarization have been demonstrated for several species, including maize (Nocito, Sacchi & Cocucci 2002; Hua *et al.* 2008; Pandolfi *et al.* 2010), barley (Chen *et al.* 2005, 2007) and *Arabidopsis* (Shabala, Shabala & Van Volkenburgh 2003; Shabala *et al.* 2006). Consistently, cytosolic K⁺ was shown to decrease rapidly upon salt treatment (in *Arabidopsis*; Shabala *et al.* 2006). The ability to retain K⁺ most effectively (i.e. to minimize K⁺ efflux when Na⁺ was administered) turned out to be a trait that correlated strongly with the ability to thrive at high salt concentrations among a collection of barley cultivars, and K⁺ flux measurements could be employed to screen for salt tolerance of a particular cultivar (Chen *et al.* 2005). Na⁺-induced efflux of K⁺ was shown to be sensitive to the K⁺ channel inhibitor Tetraethylammonium (TEA), suggesting that outward rectifying K⁺ channels are involved that are activated by the preceding membrane depolarization; this was further confirmed in direct experiments on *Arabidopsis* K⁺ transport mutants (Shabala & Cuin 2008). At a low external Ca²⁺ concentration, a fraction of the K⁺ efflux is susceptible to Gd³⁺, suggesting that non-selective cation channels (NSCCs) also provide a pathway for K⁺ efflux under these conditions (Shabala & Cuin 2008).

So far, the knowledge on transport processes induced by a sudden salt shock is largely restricted to ion exchange at the root surface, which is technically most easily accessible. However, plant adaptive response to salinity cannot be conferred to root epidermis only but should include integrated responses of numerous cell types and tissues. Studies that focus on radial transport and salt release into xylem vessels are scarce and usually deal with observations at the timescale of hours or even days (e.g. Watson, Pritchard & Malone 2001; Teakle *et al.* 2007; Shabala *et al.* 2010), with few exceptions. Among these are pulse-chase experiments with ²²Na⁺ and ⁸⁶Rb⁺ (as a tracer for K⁺) to determine the velocity at which these ions are translocated radially into the root xylem and further up into the shoot (Läuchli, Spurr & Epstein 1971; Lynch & Läuchli 1984). These studies indicated that both cations can be detected in the root xylem a

few minutes (4–5 min) after the tracers were added to the external medium. Lynch & Läuchli (1984) also used excised roots to monitor the amount of K^+ and Na^+ that was released via the cut surface, purportedly coming from the xylem vessels; for barley, they found a decrease in radial K^+ transport to the xylem immediately after the salt shock was applied.

Another approach to study the short-term effects of salt treatment is provided by the pressure probe techniques to assess radial water and solute transport parameter (Azaizeh, Gunse & Steudle 1992, Zhu *et al.* 1995; Schneider, Zhu & Zimmermann 1997; for a review, see Zimmermann *et al.* 2001). Frensch, Stelzer & Steudle (1992) challenged excised maize roots attached to a root pressure probe by adding NaCl or KCl to the external medium and inferred parameters of water and solute transport from the kinetics of root pressure changes; simultaneously, profiles of Na^+ and K^+ concentrations were obtained from root thin sections studied with the energy dispersive X-ray (EDX) technique. Pressure profiles upon addition of NaCl were biphasic, consisting of an initial pressure decrease ('water phase', because of the osmotic effect of the added salt) and a subsequent, slow recovery of the pressure that was interpreted in terms of radial diffusion of ions into the lumens of the xylem ('solute phase'). Consistently, a slow increase of Na^+ in the mature early metaxylem vessels was observed with the EDX technique; the K^+ activity remained more or less constant within 6 h after salt administration. A general objection to this experimental approach is that the experiments were performed on excised roots with extremely high salt concentrations in the xylem (~50 mM K^+), well above those found in the xylem sap of intact roots. As a result, the relevance of these data for salt effects in the intact plant appears questionable.

The drawbacks of the experimental approach of Frensch *et al.* (1992) could be overcome by making use of multifunctional xylem probes (Wegner & Zimmermann 2002, 2004, 2009; Hedrich & Marten 2006; Wegner, Schneider & Zimmermann 2007) that allow simultaneous recording of P_x , TRP and (when using double-barrelled electrodes) activities of ions (e.g. K^+ or H^+) in the xylem sap of intact, transpiring plants, rather than relying on work with excised roots. Another advantage of this technique is that the TRP is additionally measured, i.e. the electrical potential difference between the root xylem and an external reference electrode. The TRP mirrors MP changes of both cortical and stelar cells at a 1:1 relationship (Dunlop & Bowling 1971; De Boer, Prins & Zanstra 1983; Wegner *et al.* 1999) and can thus be used to monitor changes in the electrical status of both cell types.

Here, we applied this technique to investigate the effect of acute salinity stress on radial transport of K^+ . For technical convenience, most experiments were conducted on maize roots, but a few experiments on barley were also performed. Additional flux and MP measurements were undertaken using stelar segments of maize roots. Because of poor selectivity of the Na^+ liquid ion exchanger used for the fabrication of ion-selective electrodes (Chen *et al.*

2005), radial Na^+ transport could not be monitored with the probe technique. Instead, we performed Na^+ imaging using the Na^+ -sensitive dye Sodium Green. This combination of techniques enabled us to investigate early events in root ion and water transport triggered by NaCl treatment.

MATERIALS AND METHODS

Plant cultivation

Maize (*Zea mays* cv. Helix) was grown hydroponically as described previously (Wegner & Zimmermann 1998) on Rygol-Johnson medium [concentration in mM: 1.5 KNO_3 , 0.25 $MgSO_4$, 0.25 $(NH_4)_2HPO_4$, 0.25 $NH_4H_2PO_4$, 0.25 micronutrients, 0.012 Fe-ethylenediaminetetraacetic acid (EDTA)]. The calcium concentration was 2 mM (added as $CaCl_2$). Xylem probe experiments were performed on 9- to 17-day-old seedlings. The length of the main root ranged between 10 and 26 cm. Additional experiments were performed on barley seedlings (*Hordeum vulgare* cv. CM72 and Gairdner) grown under the same conditions. Plants were 10–14 d old, with the root length varying between 13 and 21 cm.

MP measurements on stelar root segments

Conventional KCl-filled Ag/AgCl microelectrodes (Shabala & Lew 2002) with a tip diameter of ~0.5 μm were used to measure NaCl-induced kinetics of MP in stelar tissues of maize. Stelar root segments were isolated and mounted in the measuring chamber as described previously for barley (Shabala *et al.* 2010). After equilibrating for 50–60 min in basic salt medium solution (BSM, in mM: 0.5 KCl, 0.1 $CaCl_2$, pH 5.6 unbuffered), steady-state MP values were measured from several root segments at three to five spots on each segment. MPs were recorded for 1.5–2 min after the potential stabilized following cell penetration. Then, with the electrode impaled, final concentrations of 20 and 100 mM NaCl, respectively, were adjusted by adding the appropriate amount of a stock solution, and transient MP changes were recorded.

Flux measurements on stelar root segments using the microelectrode ion flux estimation (MIFE) technique

Net K^+ and H^+ fluxes were measured from isolated maize stelar tissue at the mature root zone using the non-invasive ion-selective MIFE technique (UTas Innovation, Hobart, Tasmania, Australia) essentially as described in our previous publication for barley (Shabala *et al.* 2010). Root segments about 5 cm long were cut starting c. 20 mm from the root tip. The stele was extracted by gently slicing one of the cut ends and pulling apart the cortical tissue by two fine tweezers under binocular microscope. The stele was then cut into several c. 12–15 mm long segments and was left floating on the surface of the BSM solution for several hours to avoid any potential confounding effects of mechanical damage

during segments isolation. The extent of the damage was monitored in methodological experiments by measuring steady net K^+ fluxes from the isolated tissue. The damaged cells are very 'leaky' for K^+ (as one would expect from the c. 200-fold concentration gradient between the cytosol and the bath solution under our experimental conditions). The initial K^+ efflux observed immediately after the isolation winds down gradually over the next 30–40 min before stabilizing (data not shown). Hence, 2–3 h after mechanical isolation of stelar root segments was more than enough for all wounding responses to cease. Also, methodological experiments using prodidium iodide staining on mechanically isolated barley stele segment showed no evidence of cell damage (Shabala *et al.* 2010).

One hour prior to measurements, segments were fixed in a plastic holder and placed in the measuring chamber as described in our previous publication (Shabala *et al.* 2010). The steady fluxes were measured for 10–20 min to make sure that steady-state condition was reached. Then, salinity treatment was given, and transient K^+ and H^+ flux kinetics were measured for another 40–50 min. Net ion fluxes were calculated with the MIFEFLUX software (UTas Innovation), based on the measured difference in electrochemical gradient between these two positions using the cylindrical diffusion geometry as described by Shabala *et al.* (2006).

Microelectrode measurements of P_X , TRP and xylem K^+ activities

The double-barrelled multifunctional P_X probe (Wegner & Zimmermann 2002) was used to monitor kinetics of NaCl- and mannitol-induced changes in xylem K^+ concentration (A_{K^+}), P_X and TRP. Briefly, two borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) were glued together. One of them (with an inner diameter of 0.75 mm) contained a filament and was used as a K^+ -sensing barrel. Another barrel was made of a capillary with an inner diameter of 0.58 mm that contained no filament. This barrel was used for P_X and TRP measurements. After being glued together with two drops of Rislon 1000 (Risius, Pulheim, Germany), microcapillaries were pulled on a Getra vertical puller (Getra, Munich, Germany), rotating the capillaries by 360° after the first pull. The K^+ -sensing barrel was then silanized by heating the electrode for 2 h at 150 °C, backfilling the appropriate barrel with a droplet of *N,N*-dimethyltrimethylsilylamine and heating again. Silanization of the other barrel was prevented by sealing the electrode tip on a microforge (L/M CPZ101, Luigs and Neumann, Germany), and closing the open end with a drop of Silgard™ 182 (Dow Corning, Midland, MI, USA). The filling solution for the K^+ sensor contained (in % w) 18% valinomycin, 9% K^+ -tetrakis (4-chlorophenyl)borate, 25% Vinnolit™ S1565 (Vinnolit, Burghausen, Germany) and 48% 1,2-dimethyl-3-nitrobenzene. The entire mix was dissolved in four volumes of tetrahydrofuran. The backfilling solution was 10 mM KCl. The pressure/potential-sensing barrel was filled with degassed 50 mM *N*-methyl-D-glucamine-2-(*N*-morpholino)

ethanesulphonic (NMG-MES) (pH 5.8) as described previously (Wegner & Zimmermann 1998).

Before measurement, the electrode tip was broken to achieve the required diameter, and the blunt end of the pressure/potential-sensing barrel was attached to the xylem pressure/potential probe as described previously (Wegner & Zimmermann 2002). The probe was then mounted on a micromanipulator (Leica, Bensheim, Germany), and an Ag/AgCl electrode was inserted into the K^+ -selective barrel via the open, blunt end and connected to a high-impedance electrometer (FD223 from World Precision Instruments). P_X , TRP and K^+ potential data were fed, via an analogue to digital converter (DAS 1601, Keithley, Taunton, MA, USA), into a personal computer (Pentium 133), using Testpoint™ software (Keithley), at a sampling rate of 10 Hz, and were filtered at 0.1 Hz. The K^+ electrode was calibrated in a set of appropriate standards as described elsewhere (Wegner & Zimmermann 2002).

During measurement, hydroponically grown maize or barley roots of 9- to 17-day-old plants were mounted on a Perspex holder and were immersed in a bath solution containing (if not stated otherwise) 1 mM KCl, 2 mM $MgCl_2$, 0.1 or 2 mM $CaCl_2$, 10 mM MES/BTP (pH 5.5). The root was allowed to equilibrate with the medium under laboratory conditions for ~1 h, and the multifunctional probe was inserted into a single xylem vessel of a seminal root by slowly advancing the tip of the probe through the tissue as described elsewhere (Wegner & Zimmermann 1998, 2002). P_X , A_{K^+} and TRP values were measured for 20–30 min to ensure they were stable, then, an appropriate concentration of NaCl or mannitol was added, followed by another 40–80 min of recording. These experiments were performed at a low light irradiation (~10 $\mu mol photons m^{-2} s^{-1}$). When the P_X still remained above vacuum even after the osmoticum had been administered, irradiation was increased to about 300 $\mu mol m^{-2} s^{-1}$ to shift P_X to negative values (Wegner & Zimmermann 1998); when P_X did not drop below vacuum at the elevated light regime, the experiment was discarded, assuming that the probe contained an air bubble and/or that the root had become leaky by impalement.

Imaging of radial Na^+ transport into the stele

Roots were incubated for 1 h in 1 mL of 10 μM membrane impermeant tetra (tetramethylammonium) salt of Sodium Green indicator (S-6900, Molecular Probes, Eugene, OR, USA) prepared in BSM media (S-6900, Molecular Probes). After 30, 55 or 59 min in Sodium Green, 100 mM NaCl was added to the solution containing the probe so that the final time of exposition to salt was 30, 5 or 1 min. Roots were then quickly but thoroughly rinsed in 5 mM $CaSO_4$, blotted with a paper towel, and stelar segments were mechanically isolated as described above.

After 1 h of incubation, the samples were examined using confocal microscopy. Images were recorded with a Leica TCS SP5 confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany), equipped with an

acousto-optical beam splitter (AOBS), and an upright microscope stand (DMI6000). The microscope setting for detecting Sodium Green was $\lambda_{exc} = 488$ nm and λ_{em} over the 520–560 nm spectral band. A series of confocal optical XY images through the thickness of the samples (total scanning volume was ~ 100 μm , with a slice thickness of 5 μm) were acquired in XYZ scanning mode, using the Leica LAS AF software package (Leica Microsystems CMS GmbH). Comparison of different levels of fluorescence between stellar thin sections was carried out by visualizing with the identical imaging settings of the confocal microscope (i.e. laser intensity, pinhole diameter and settings of the imaging detectors).

RESULTS

Xylem K^+ probe measurements

In order to investigate the short-term effects of salinity stress on P_x , on xylem K^+ activity (A_{K^+}) and on radial electrical potential gradients (TRP) in roots, a multifunctional xylem K^+ probe was employed (see Introduction).

Due to methodological reasons, the majority of the experiments were performed on maize roots while a few were also conducted on barley. A representative example of NaCl-induced changes in P_x , A_{K^+} and TRP is shown in Fig. 1a. This experiment was performed on a 10-day-old maize seedling; the root was immersed in a bath medium containing 1 mM KCl and 0.1 mM CaCl_2 (low Ca). Under control conditions (no salt treatment; ambient light of ~ 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), a sub-atmospheric pressure of ~ 93 kPa was recorded. Upon addition of 100 mM NaCl to the bath, the P_x dropped with an exponential time course to 51 kPa because of water efflux from the root elicited by the increase in external osmotic pressure (by 457 kPa). When P_x had attained a steady value, light irradiation was increased to ensure that pressure responded to light and negative pressure in the impaled xylem vessel and in the probe could be established (at $t = 102$ min, data not shown); indeed, pressure dropped below vacuum with a few minutes delay as previously described in detail (Wegner & Zimmermann 1998, 2002, 2009; Shabala *et al.* 2009).

Simultaneously, the response of the TRP following the addition of NaCl was monitored (Fig. 1a). This parameter reflects MP changes both of cortical and of stellar cells (Wegner *et al.* 1999). Under control conditions, a steady value of -35 mV was recorded in this particular experiment. Salt treatment elicited a rapid depolarization to -3 mV (within 65 s) followed by a slower recovery of the TRP almost to the original value (-30 mV). The transient excursion of the TRP was completed after about 7 min. Subsequently, it remained constant until light irradiation was increased.

The third parameter to be continuously monitored was the xylem K^+ activity; it was 6.9 mM under control conditions. When NaCl was added, the xylem K^+ activity dropped transiently by 2.4 mM and increased again to 5.9 mM. In this experiment, a final value of 4 mM was reached about 20 min

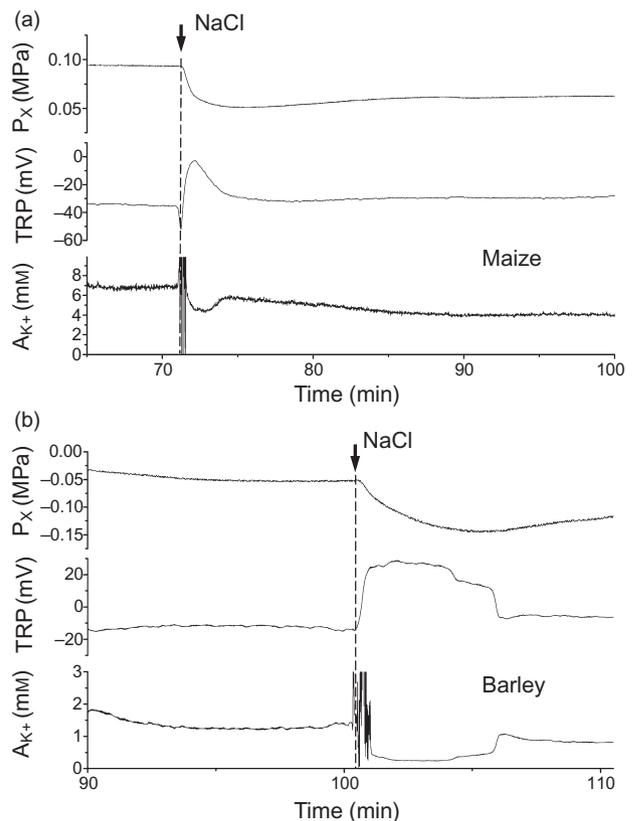


Figure 1. Salinity (100 mM NaCl)-induced changes (one of four representative examples) in the xylem K^+ activity (A_{K^+}), P_x and TRP in the main root of a 10-day-old maize plant (a) and a root of a 14-day-old barley plant (cv. CM72; b) immersed in low- Ca^{2+} (0.1 mM) bath solution. Note that P_x in the maize root dropped below zero when photon flux density was subsequently increased from about 10 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (not shown).

after NaCl addition; in six other experiments (out of $n = 8$), A_{K^+} returned to the value measured before exposure to salt. A transient drop of xylem K^+ was observed in seven out of eight experiments. In one experiment, a transient increase in K^+ was elicited by salinity.

Results of the experiments performed with the multifunctional probe are summarized in Table 1; note that the experiments were performed at two external Ca^{2+} levels (0.1 mM – low, 2 mM – high) because Ca^{2+} is known to interfere with the response of roots to external Na^+ (Lynch, Cramer & Läuchli 1987; Mansour 1995; Shabala *et al.* 2003, 2006). Varying the Ca^{2+} level in the bath solution had no significant ($P < 0.05$) effect on A_{K^+} . Interestingly, however, TRP values under control conditions were more negative (by 20–25 mV) at low Ca^{2+} as compared with high calcium in the bath (significant at $P < 0.05$). At high Ca^{2+} , the amplitude of the rapid depolarization was less than the subsequent repolarization leading to a steady-state TRP value that was more negative after challenge with a salt shock than before. An overview on the kinetics of salt-induced TRP changes is given in Table 2. Interestingly, depolarization was somewhat slower at the high compared with the low external Ca^{2+} concentration, as indicated by the

Parameter	Before treatment	After treatment	Peak change
Low Ca			
P_X (kPa)	76 ± 8.4 ^b (7)	65 ± 5.4 ^b (7)	-24.7 ± 6.3 ^b (7)
TRP (mV)	-36 ± 3.9 ^a (6)	-29 ± 4 ^a (7)	35.3 ± 4.1 ^a (7)
A_{K^+} (mM)	5.5 ± 1.4 ^b (3)	4.71 ± 1.42 ^b (3)	-1.64 ± 0.3 ^b (3)
High Ca			
P_X (kPa)	58 ± 9.5 (4)	38 ± 13 (6)	-24 ± 7 (4)
TRP (mV)	-0.7 ± 2.3 (5)	-9.9 ± 2.6 (5)	14.4 ± 3.2 (4)
A_{K^+} (mM)	6.2 ± 0.4 (4)	4.77 ± 0.39 (4)	-3.0 ± 0.66 (4)

Mean ± SE (n = sample size).

^aIndicates significant difference (at $P < 0.001$) for an appropriate parameter between low- and high-Ca treatment; ^bnot significant at $P < 0.05$.

Parameter	Characteristic time points of TRP response		
	TRP maximum	Half-maximum depolarization	Half-maximum repolarization
Low Ca			
Time after addition of NaCl (s)	51.6 ± 10.9 ^a (6)	18.5 ± 2.3 ^a (6)	395 ± 174 ^a (6)
Slope (mV/s)		1.91 ± 0.25 ^b (6)	-0.11 ± 0.04 ^a (5)
High Ca			
Time after addition of NaCl (s)	56.4 ± 14.4 (4)	29.4 ± 7.7 (4)	200 ± 62 (3)
Slope (mV/s)		0.68 ± 0.15 (3)	-0.15 ± 0.09 (3)

Parameters describing the time course of TRP changes in the main root of 10- to 15-day-old maize roots (compare Fig. 1) at the low and high external Ca-concentration (0.1 and 2 mM, respectively) are summarized (mean ± SE, number of replicates in brackets).

^aNot significant at $P < 0.05$ compared with high-Ca treatment. ^bSignificant at $P < 0.05$ compared with high-Ca treatment.

difference in the slope of the TRP recordings (0.68 versus 1.91 mV/s) at half-maximum depolarization.

P_X values were also slightly (by ~20 KPa) lower with the higher Ca^{2+} concentration before salt treatment, reflecting the minor difference in the osmotic pressure of the bath.

When measurements of P_X and TRP were repeated using a single-barrelled xylem pressure/potential probe filled with 50 mM KCl instead of NMgCl ($n = 3$ experiments with low Ca^{2+}), the same response to NaCl treatment, as shown in Fig. 1a, was found, including the characteristic transient xylem depolarization (no significant difference at $P < 0.05$). This indicates that contamination of the xylem sap by the electrode filling solution did not affect the experimental result; as a consequence, P_X and TRP data measured with single- and double-barrelled electrodes were pooled.

Qualitatively similar results were also obtained on barley seedlings. In the experiment shown in Fig. 1b, the acute salt shock induced a decrease in P_X by 91 kPa (from -52 to -143 kPa, preceded by a rapid TRP depolarization by 42 mV (on average, 46 mV ± 4 mV SE; $n = 3$). The TRP remained in a depolarized state for about 2 min; subsequently, a repolarization of the xylem started at about the same rate as the depolarization. K^+ activity dropped from 1.4 to 0.25 mM and recovered partly after about 6 min (final value 0.82 mM).

To distinguish between osmotic and ion-specific effects of salt stress on TRP and A_{K^+} , the experiment was repeated,

Table 1. Salinity (100 mM NaCl)-induced changes in the P_X , TRP and xylem K^+ activity (A_{K^+}) measured in the main root of 9- to 17-day-old maize seedlings at two Ca^{2+} levels (low, 0.1 mM; high, 2 mM) in the bath solution

Table 2. Kinetics of the TRP changes induced by administration of 100 mM NaCl

adding mannitol to the bath instead of NaCl at the low-Ca regime. A typical experiment out of four replicates performed on maize is shown in Fig. 2. Upon addition of 170 mM mannitol (isotonic to 100 mM NaCl; Schneider *et al.*

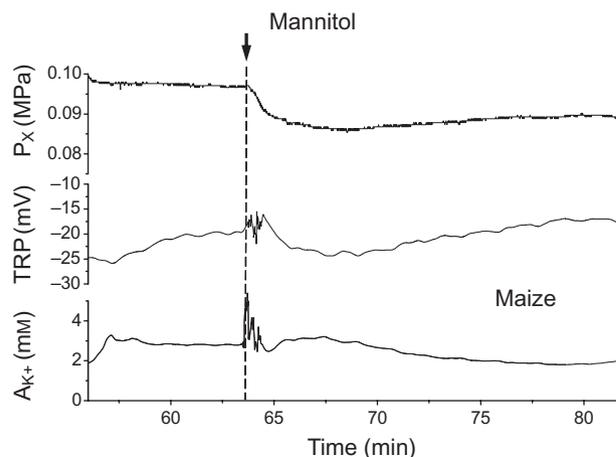


Figure 2. Mannitol-induced changes in the xylem K^+ activity (A_{K^+}), P_X and TRP in maize roots. One (of four) representative examples are shown; 170 mM mannitol (isotonic to 100 mM NaCl) was added at the time indicated by the arrow. Note that P_X dropped below zero when the light intensity was subsequently increased from 10 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (not shown).

1997), the P_x responded in the same way as described for NaCl, as expected for a pure osmotic effect. However, in contrast to the transient depolarization induced by NaCl treatment, addition of mannitol elicited a transient hyperpolarization (in this experiment, by up to -4 mV recorded after ~ 5 min). On average, the initial TRP was -20 ± 4.7 mV (mean value \pm SE) for this batch of maize seedlings; mannitol-induced hyperpolarization was quite variable, with a maximum amplitude of up to -27 mV. Steady-state TRP values in the presence of mannitol were less negative than those measured before the osmoticum was applied. The K^+ activity remained between 2 and 4 mM and was largely unaffected by root exposure to mannitol.

NaCl-induced changes in the MP of stelar cells in maize

The characteristic profile of the TRP induced by salt treatment resulted from changes in MPs of both cortical and stelar cells. While data on the effect of NaCl on cortical MPs in maize roots have been reported before (Hua *et al.* 2008), no information is available for stelar cells. Unfortunately, stelar MPs are not accessible on intact roots; instead, we performed experiments on isolated steles. We also measured NaCl-induced changes in net K^+ (and H^+) fluxes at the surface of stelar segments to shed more light on the transient drop in xylem K^+ after salt treatment.

Under control conditions (in the absence of NaCl in the bath), the MP of maize stelar cells was around -120 mV (Fig. 3a & b; open bar). Adding 20 mM NaCl (a Na^+ concentration that is not uncommon for the xylem of a plant exposed to salt stress; Shabala *et al.* 2010) rapidly (within 1–1.5 min; Fig. 3a) depolarized the plasma membrane of stelar cells by 20–25 mV (Fig. 3a & b) within 1–1.5 min. At a higher NaCl concentration (100 mM), the depolarization was even more pronounced (Fig. 3c; records #3 to #9). All effects were significant at the $P < 0.01$ level.

NaCl-induced changes in net K^+ and H^+ fluxes at the surface of stelar segments isolated from maize roots

Measurements of ion fluxes at the surface of stelar segments with the MIFE technique were also performed (Fig. 4). Experimental conditions corresponded to those established for recordings of MPs. When 20 mM NaCl was added to the bath, K^+ efflux was elicited instantaneously (within the limits of accuracy of the method; Fig. 4a, significant at $P < 0.01$). Subsequently, efflux increased slightly further. Proton efflux was also transiently enhanced by salt treatment at a similar time course (Fig. 4b). Addition of 100 mM NaCl caused a large K^+ efflux that decreased with time (within about 10 min) to the same level as measured with the lower concentration. Parallel to K^+ efflux, large H^+ efflux was elicited that decreased, albeit at a slower rate, to the efflux regime measured with 20 mM NaCl.

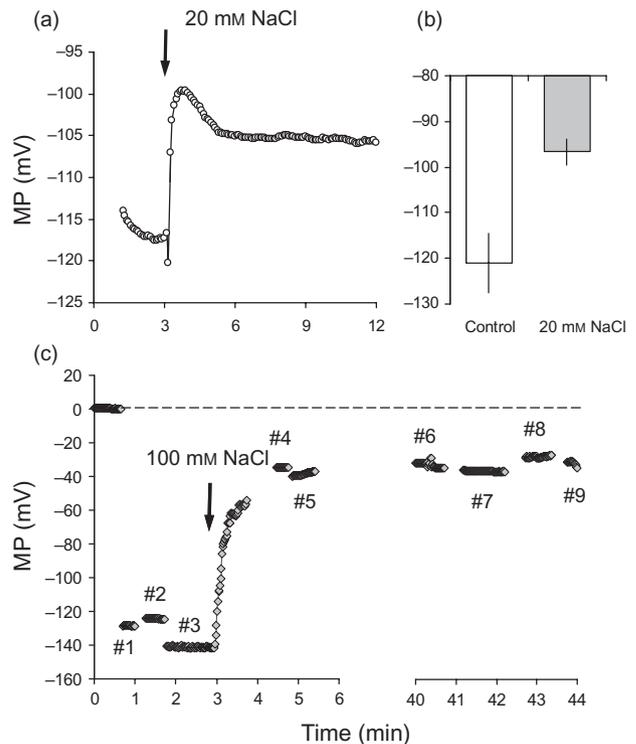


Figure 3. NaCl-induced changes in MP of maize stelar cells. (a) Transient MP kinetics in response to 20 mM NaCl treatment. One (of four) typical example is shown. (b) Steady-state MP values before and after 20 mM NaCl treatment. Mean \pm SE ($n = 6$). (c) Transient MP kinetics in response to 100 mM NaCl treatment. Measurements from nine different spots (labelled by #) from three individual roots are combined in one panel.

Radial Na^+ transport in maize roots

The maize roots were treated with the membrane impermeant tetra (tetramethylammonium) salt of sodium Green dye (Fig. 5b). The dotted lines in this panel illustrate a position of the xylem vessels, and it is obvious that fluorescent signal is absent from these parts of the stele. On the contrary, after 5 min of exposure of intact roots to 100 mM NaCl, a substantial amount of Na^+ was released into the xylem vessel (Fig. 5c) and even stronger fluorescent signals were measured after 30 min NaCl exposure (Fig. 5d). The above experiment was repeated 12 times, always yielding a consistent result: no detectable fluorescent signal related to Na^+ was observed in the xylem vessel for control and 1 min NaCl treatment. The signal became noticeable after 5 min, and the maximum fluorescence intensity was always detected for 30 min NaCl treatment. Thus, it appears that the Na^+ loading into the xylem

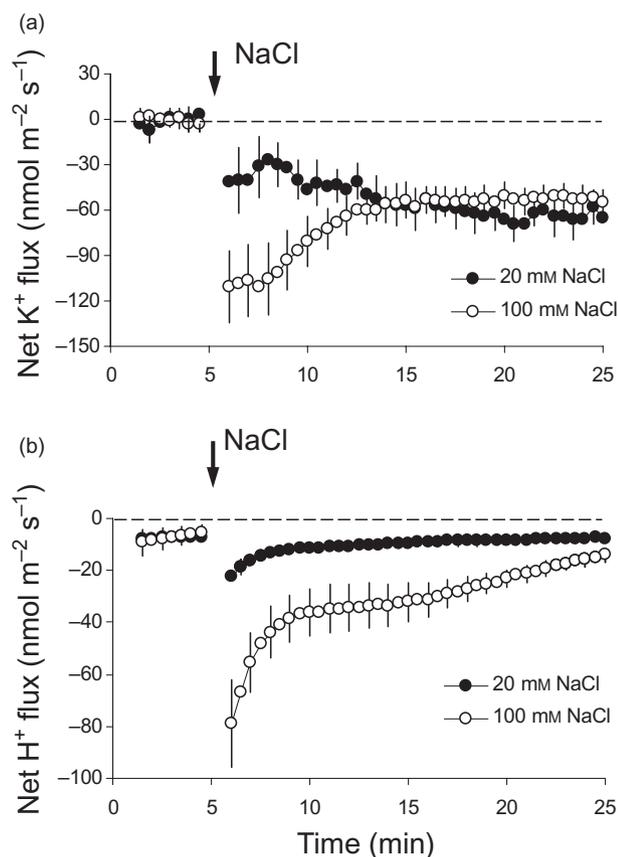


Figure 4. Kinetics of net K⁺ (a) and H⁺ (b) fluxes measured from maize stele in response to 20 mM (closed symbols) or 100 mM (open symbols) NaCl. Mean \pm SE ($n = 5-8$). The sign convention is 'efflux negative'.

starts in the time interval between 1 and 5 min of root exposure to the salt stress.

DISCUSSION

It was reported before that mechanical separation of cortex and stele occurs at the endodermis, which is destroyed by this process in maize (Hall, Sexton & Baker 1971). This issue was further investigated in detail by Shabala *et al.* (2010), showing that both MP and ion flux measurements on isolated stelar segments reflected properties of xylem parenchyma rather than endodermal cells. Hence, with a high degree of confidence, electrophysiological data on isolated stelar tissue referred here can be attributed to the xylem parenchyma cells.

In stelar segments isolated from maize roots, an acute salt shock (20 or 100 mM NaCl) elicited a rapid K⁺ and H⁺ efflux (Fig. 4). With 100 mM NaCl added to the bath, K⁺ efflux decreased within minutes by about 50%; a residual component prevailed until the end of the experiment (about 20 min after salt addition). This response was quantitatively and qualitatively very similar to the one observed for roots of intact maize seedlings (Pandolfi *et al.* 2010), with efflux originating at rhizodermal and cortical, instead of stelar,

cells. Consistently, NaCl treatment caused a sustained depolarization of parenchyma cells both in the isolated steles of maize roots (Fig. 3) and in the root cortex (Hua *et al.* 2008). Apparently, a common mechanism underlies the susceptibility of both cortical and stelar cells to a salt shock. This implies that both cell types are equipped with a similar set of transporters that are involved in the response to salt. Patch clamp experiments on protoplasts isolated from cortical and stelar cells confirmed that this is indeed the case, at least with respect to cation-selective channels. Indeed, outward rectifying K⁺ channels and NSCCs were found in both cell types (Roberts & Tester 1995; Zhao *et al.* 2007; but see also Diatloff *et al.* 2004 for differences in channel properties). The flux and MP data reported here for maize roots agree well with those previously published for barley (Shabala *et al.* 2010). For that species, pharmacological data on Na⁺-induced K⁺ efflux are also available for both intact roots (Chen *et al.* 2007) and for stelar segments (Shabala *et al.* 2010). For the segments immersed in a medium containing 1 mM Ca²⁺, it was shown that TEA, a potent blocker of the K⁺ outward rectifier in barley xylem parenchyma (Wegner & Raschke 1994; Wegner & De Boer 1997) eliminated K⁺ efflux almost completely, indicating that the transient efflux component was predominantly mediated by this type of ion channel. By contrast, addition of an NSCC blocker, Gd³⁺, had no effect.

However, it is not clear to what extent properties of stelar cells change after removal of the root cortex (Laties & Budd 1964; Yu & Kramer 1969). Moreover, stelar cells do not usually experience a sudden increase in external NaCl, as rhizodermal and cortical cells do; rather, apoplastic Na⁺ in the stele will increase gradually with time. In this study, we used the Na⁺-selective dye Sodium Green to monitor the Na⁺ increase in the xylem sap and in the stelar apoplast of intact roots when exposed to a sudden salt shock. Few studies have been published so far that make use of Na⁺-selective dyes for measuring intracellular (Halperin & Lynch 2003) or apoplastic Na⁺ (Mühling & Läuchli 2002). Here, it was found that after 1 min of salt exposure, no signal was detectable in the vessels themselves (Fig. 5b), although preliminary experiments with membrane-permeant dye suggested that some Na⁺ had already reached the parenchyma cells bordering on xylem vessels (data not shown). After 5 min though, a Na⁺ signal was clearly detectable in the xylem vessels (Fig. 5c) and became stronger after 30 min of salt exposure (Fig. 5d). This is in a good agreement with pulse-chase experiments using ²²Na⁺ (Läuchli *et al.* 1971); however, in the latter study, roots had been pre-treated with Na⁺ for several hours; hence, results are not directly comparable. Na⁺ transport into the xylem involves both apoplastic and symplastic pathways (Azaizeh *et al.* 1992; Shabala *et al.* 2010). Symplastic transport ends at the plasma membrane of stelar parenchyma cells; passage into the vessels occurs via transport proteins that are permeable to Na⁺, e.g. the Na⁺/H⁺ antiporter SOS1 (Shi *et al.* 2002). Obviously, this final membrane transport step is rate-limiting for Na⁺ transport into the xylem.

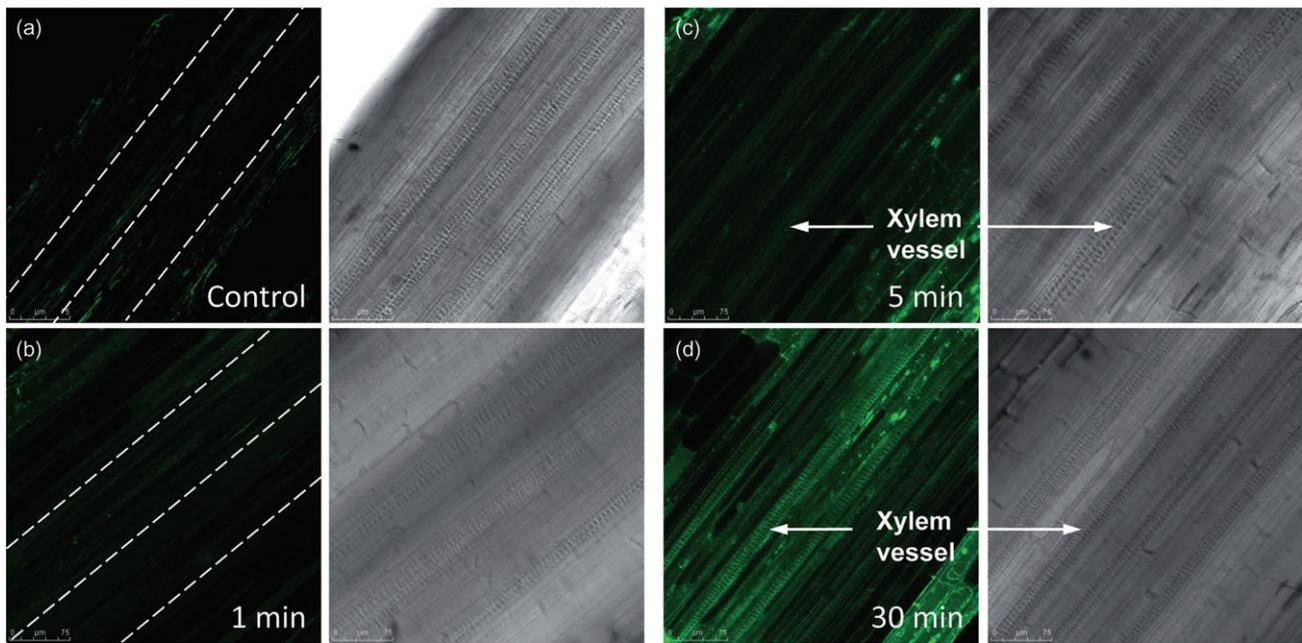


Figure 5. Xylem Na^+ loading in maize roots visualized by confocal microscopy using Sodium Green dye. Maize roots were treated with 100 mM NaCl for various amounts of time (from 0 – control – to 30 min). One of 12 representative examples is shown. Roots were stained with 10 μM Sodium Green dye for 1 h. After 30, 55 or 59 min in Sodium Green, 100 mM NaCl was added to the solution containing the probe so that the final time of exposition to salt was 30, 5 or 1 min. Roots were then quickly, but thoroughly, rinsed in 5 mM CaSO_4 , and the confocal images were then taken. Measurements were made in the mature zone, between 10 and 20 mm from the root apex. Each panel consists of a fluorescent micrograph (left) and the corresponding light micrograph (right). Dotted lines in panels (a) and (b) indicate position of the xylem vessels.

Uncertainties in the interpretation of electrophysiological data obtained on isolated steles (and on excised roots; see Introduction) could be overcome by making use of the multifunctional xylem K^+ probe (Wegner & Zimmermann 2002, 2009; Wegner *et al.* 2007). With this tool, the TRP can be measured, which reflects the difference in the MPs of both cortical and stelar cells (provided that the transpirational water flow is absent or very low; De Boer *et al.* 1983; Wegner *et al.* 1999), and can thus be used to monitor changes in the MP of both cell types in an intact, transpiring plant. It is important to note that changes in the stelar MP contribute to the TRP with opposite polarity, i.e. a depolarization of stelar parenchyma corresponds to a negative shift in the TRP (De Boer *et al.* 1983). Upon an acute salt shock, we observed a rapid depolarization of the xylem vessel with respect to the bath (see Table 2) that closely resembled the response of the cortical MP (Hua *et al.* 2008). Subsequently, however, a repolarization of the TRP occurred, which was absent in cortical MP recordings on maize (Hua *et al.* 2008), on barley (Chen *et al.* 2007), and on *Arabidopsis* (Shabala *et al.* 2006). Apparently, the second phase of the TRP response reflected a depolarization of stelar cells (the MP of which is not directly accessible on intact roots) that started with a delay of about 60 s (Table 2); during this time interval, some Na^+ had most likely arrived in the stelar symplast, but apoplastic Na^+ was still below the detection limit (Fig. 5b). Hence, it appears that depolarization of stelar parenchyma cells (corresponding to a repolarization

of the TRP) is initiated by intracellular, rather than apoplastic, Na^+ . However, the further time course of repolarization, which was about 10-fold slower than the preceding depolarization (compare slope data in Table 2), is probably governed by the rate of Na^+ accumulation in the stelar apoplast.

Apparently, at the low external Ca^{2+} concentration, both cell types were depolarized roughly to the same extent (Fig. 1a, Table 1), and hence, the effects of cellular MPs on the TRP cancelled each other out; indeed the TRP remained constant after returning to the original level. Consistently, Bowling & Ansari (1972) reported that NaCl did not affect steady-state TRP values in excised sunflower roots.

Besides measuring the TRP, the probe technique allows continuous recording of the xylem K^+ activity and, hence, renders information on radial K^+ transport into the stele. Interestingly, a transient decrease in the xylem K^+ activity was observed in seven out of eight experiments performed on maize seedlings; and this also occurred in barley (Fig. 1; Table 1). This result appears to be at variance with the K^+ flux data obtained on isolated steles that would, at first glance, suggest a transient increase in xylem K^+ , rather than a decrease. Such increase in xylem K^+ concentrations was reported in experiments with salt-grown barley plants using Scholander pressure bomb (Shabala *et al.* 2010). The physiological rationale behind this increase is the maintenance of a favourable K^+/Na^+ ratio in the shoot while plants increase

the uptake of these inorganic osmotica for shoot osmotic adjustment purposes and maintenance of the shoot's growth.

Previous experiments have clearly shown that the probe is capable of accurately recording changes in K^+ activity even during rapid TRP changes (e.g. after root cutting close to the site of probe insertion; Wegner & Zimmermann 2002); the error caused, e.g. by a delayed response of the K^+ -sensing barrel to a TRP change (Felle & Bertl 1986), will be negligible in the recordings presented here. Our data indicate that a massive salt shock elicits a reabsorption of K^+ from the stelar apoplast immediately after treatment, which is not observed on isolated steles. One scenario to reconcile probe and stelar flux data is related to the massive K^+ efflux from the root symplast via the plasma membrane of cortical cells in the intact root, which precedes depolarization of the cortical cells. For *Arabidopsis*, it has been shown that a treatment of the root with moderate NaCl concentrations (50 mM) led to a rapid (within 10 min) decline of the cytosolic K^+ activity (Shabala *et al.* 2006). No data are available for maize roots, but disturbance of K^+ homeostasis by a salt shock seems to be equally severe in both species, as judged from the amount of K^+ efflux per root surface. Therefore, cytosolic K^+ is likely to respond in a similar way in both species (unless the capacity of the vacuoles for buffering cytosolic K^+ is higher in maize than in *Arabidopsis* roots). As long as the plasma membrane of stelar parenchyma cells remains in a hyperpolarized state, a decrease in symplastic K^+ will transiently shift the electrochemical potential gradient towards K^+ resorption from the stelar apoplast (including xylem vessels) by xylem parenchyma cells. This gradient is reversed again when depolarization of xylem parenchyma cells starts (corresponding to the second phase of the TRP response to an acute salt shock) as soon as Na^+ arrives at the root centre, i.e. from 1 min after salt application onwards. Alternatively, the transport capacity of stelar cells for K^+ uptake could have increased transiently when the root was challenged with a salt shock. In any case, subsequent membrane depolarization entailed K^+ efflux from xylem parenchyma cells, in agreement with the flux data obtained on stelar segments. As a consequence, the K^+ activity in the xylem recovered, at least transiently.

Interestingly, after the perturbation, the xylem K^+ activity equaled the value measured before the salt shock or was only moderately lower; this indicates that the decrease in cytosolic K^+ activity is, at least, partly compensated by an increase in K^+ channel activity following membrane depolarization (Roberts & Tester 1995). This conclusion is also supported by EDX data on maize roots (Frensch *et al.* 1992), indicating that within 4 h after the salt shock, the K^+ concentration decreased in tissue cells but remained constant in the mature early metaxylem vessels that mediated long-distance transport to the shoot.

Our data suggest that the electrophysiological response of the root to an acute salt stress was specific to NaCl and not because of an unspecific effect of increasing the osmotic pressure of the bath. The osmotic effect of adding

100 mM NaCl ($\Delta\pi = 187 \text{ mosmol kg}^{-1}$, corresponding to 457 kPa) to the bath manifested itself in a hydrostatic pressure drop by 25 kPa. The apparent radial reflection coefficient, σ_r , of the root was calculated to be about 0.055; Frensch *et al.* (1992) using the root pressure probe, obtained a σ_r -value of 0.56. The relatively weak response of P_x to an osmotic shock in the intact plant is not due to a leak caused by the insertion of the probe, as subsequent increase in light irradiation caused a pressure drop by several hundred kilopascals, well below vacuum (data not shown). Rather, it may be related to the fact that the NaCl concentration used here was higher than in the study by Frensch *et al.* (1992; 40–70 mosmol kg^{-1} NaCl). Recently, Bai *et al.* (2007) reported for *Atriplex triangularis*, a halophyte, that σ_r -values decreased dramatically with the NaCl concentration applied externally. For a more detailed discussion of this topic, see Schneider *et al.* (1997), Zimmermann *et al.* (2001) and Bai *et al.* (2007). Interestingly, the hydrostatic pressure response of barley roots to the same osmotic treatment was more pronounced (Fig. 1b), with σ_r ranging from 0.11 to 0.31, consistent with Schneider *et al.* (1997).

In intact maize plant, a similar drop in P_x was evoked by adding an isotonic concentration of mannitol to the bath instead of NaCl. However, a different pattern of the TRP response was found, and, in contrast to the salt effect, xylem K^+ remained largely unaffected. Initially, the osmotic shock caused a moderate hyperpolarization of the TRP that obviously mirrored a corresponding change in the MP of cortical cells (Ober & Sharp 2003), apparently being part of the osmoregulation process (Shabala & Lew 2002). This type of response appears to be independent of the applied osmoticum. When NaCl was administered, however, the osmotic response was masked by the dominating effect of the particular ions, particularly of Na^+ (reviewed in Shabala & Cuin 2008); this effect may be even more pronounced when NaCl is supplied while keeping the effective osmotic pressure of the bath at a constant level.

It may be questioned whether short-term salt effects on physiological parameters of crop plants are relevant for agricultural management; under field conditions, salt stress will develop rather gradually, with salt concentrations in soil increasing at the time scale of hours or even days. However, Lynch & Läuchli (1984) argue that acute salt stress may occur with poor quality irrigation water, and saline agriculture is gaining momentum in light of the persistent drought problems in many parts around the world (Robinson *et al.* 2004; Grattan *et al.* 2008). Apart from this, a precise understanding of the first steps initiated when the plant is confronted with high concentrations of NaCl will make an important contribution to unraveling the complex physiology of salt stress.

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