Sodium Distribution in Salt-stressed Citrus Rootstock Seedlings

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Abstract. Although citrus trees are considered relatively salt-sensitive, there are consistent differences in Na⁺ and Cl[−] tolerance among different citrus rootstocks. We grew uniform seedlings of rough lemon (RL) and the more Na⁺-tolerant Swingle citrumelo (SC) with and without 50 mM NaCl for 42 days. Salinity reduced leaf chlorophyll and plant transpiration rate (E_p) more in RL than SC. Confocal laser scanning analyses using the Na⁺-specific cell-permeant fluorescent probe CoroNa-Red revealed a higher capacity for Na⁺ sequestration in root tissue vacuoles of SC than in RL roots and that cell walls within the stele acted as Na⁺ traps. In leaves, however, RL had significantly higher Na⁺-dependent fluorescence than SC. Thus, the sequestration of Na⁺ in root tissue vacuoles and its immobilization by cell walls were key contributing mechanisms enabling SC leaves to maintain lower levels of Na⁺ than RL leaves. Examination of intracellular distribution of CoroNa-Green fluorescence in SC root protoplasts verified a vacuolar localization for Na⁺ in addition to the presence of a 2- to 6-μm unidentified endosomal compartment containing significantly higher Na⁺ concentrations.

Although citrus trees are considered relatively salt-sensitive, there are inherent differences in Na⁺ and Cl[−] tolerance among the many commercial citrus rootstocks (Castle et al., 2006; Maas, 1993). Salinity tolerance of citrus rootstocks is gauged by satisfactory tree growth and fruit yield under saline conditions (Castle et al., 1993) along with the amount of Cl[−] and/or Na⁺ accumulated in foliage (Levy and Syvertsen, 2004; Maas, 1993). There are often conflicting interpretations of the relative levels of rootstock tolerance to salinity depending on variations in salinity treatments, treatment duration, scion type, growth substrate, and which type of physiological responses were measured (Levy and Syvertsen, 2004; Zekri and Parsons, 1992).

Salt tolerance represents the natural ability to cope with absorbed salt ions usually involving the chelation, exclusion, or sequestration of toxic concentrations of salts away from the photosynthetic machinery in leaves (Munns and Tester, 2008). Because citrus trees are usually grafted onto rootstocks, these trees represent an opportunity to study their ability to restrict the transfer of salt ions to the shoot when used as rootstocks. Rootstock characteristics tend to be consistent even when grown as seedlings in a greenhouse (Syvertsen et al., 2010). Citrus and other fruit trees are somewhat unique in that Cl[−] ions are considered to be more toxic than Na⁺ ions (Levy and Syvertsen, 2004). Thus,

salt tolerance in citrus is usually based on Cl[−] toxicity (Maas, 1993), but Cl[−] toxicity is seldom studied in the absence of Na⁺. Although the allocations of Na⁺ and Cl[−] at the organ level are well described among citrus rootstocks with varying salt tolerances (Garcia-Sanchez and Syvertsen, 2006; Zekri and Parsons, 1992), the fundamental nature of the exclusion, sequestration, and/or compartmentation at the tissue and cell levels remains unresolved. Our preliminary dye fluorescence studies on Na⁺ and Cl[−] distribution in citrus tissues revealed that Na⁺ dyes are more effective than Cl[−] dyes in their fluorescence intensity and stability properties. Thus, as a first step toward understanding salt tolerance and salt ion distribution in citrus tissues, we studied Na⁺ distribution in root and leaf tissues of seedlings of citrus rootstocks with contrasting tolerances to Na⁺ ions. Despite the critical importance of active Na⁺ efflux systems for salinity tolerance in many plant species (see review by Munns and Tester, 2008), such systems have not been characterized in salt-sensitive citrus trees.

Na⁺ tolerance can be achieved by reducing Na⁺ uptake by the roots, sequestration within the root tissues, decrease loading to the xylem, or by an enhanced capacity of intracellular compartmentation of Na⁺ in leaf cells (Hasegawa et al., 2000; Tester and Davenport, 2003). In addition, large amounts of ions could be excluded from the metabolic milieu by sequestration in the apoplast as reported for metal ions (Mari and Lebrun, 2006). In both roots and shoots, metabolic activities that impart salt tolerance require coordination between cell and tissue types mostly mediated by membrane-bound carriers

and transporters (Cuin et al., 2011). Important mechanisms that contribute to Na⁺ tolerance are the membrane-localized Na⁺ transporters at the tonoplast and the plasmalemma (Tester and Davenport, 2003). Tonoplast-bound Na⁺/H⁺ antiporters mediate the removal of Na⁺ into the vacuole. Overexpression of Na⁺/H⁺ antiporters in *Arabidopsis* (Shi et al., 2003), Brassica (Zhang et al., 2001), and tomato (Zhang and Blumwald, 2001) has resulted in significant improvements in salt tolerance. Using a variety of fluorescent dyes, Hamaji et al. (2009) demonstrated the almost exclusive localization of Na⁺ in the vacuolar space of root cortical cells and in root suspension cultured cells of *Arabidopsis*.

Removing excess Na⁺ from the cytosol can also be accomplished by plasmalemma-bound Na⁺ efflux transporters that extrude Na⁺ to the apoplastic space (Shi et al., 2002). The use of the apoplast as a depository of surplus or toxic ions has been demonstrated for metal ions such as nickel in roots of *Leptoplax* (Redjala et al., 2010). The ability of a salt-tolerant wheat cultivar, Kharchia 65 (Cuin et al., 2011), to extrude Na⁺ from the roots may contribute to its salinity tolerance. However, this strategy may be only delaying the transfer of toxic levels of Na⁺ from roots to shoots.

At the whole plant level, different citrus rootstock species can differ in their allocations of Na⁺ between roots and shoots (Garcia-Sanchez and Syvertsen, 2006, 2009). Although the common citrus rootstock rough lemon (RL) (*Citrus jambhiri* Lush.) and Swingle citrumelo (SC) [*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf] are both considered salt-sensitive based on Cl[−] toxicity, SC (and other *P.t.* hybrid rootstocks) has the ability to limit Na⁺ accumulation in leaves compared with RL (Levy and Syvertsen, 2004). The mechanism of how such a difference in Na⁺ distribution occurs in trees grafted on these contrasting rootstocks could be related to differences in Na⁺ sequestration in their root tissues. Thus, based on molecular analyses in *Arabidopsis* (Shi et al., 2003; Zhu, 2000) and on determinations of intracellular Na⁺ distribution with Sodium-Green (Hamaji et al., 2009), we hypothesized a higher Na⁺ sequestration in the root vacuoles of the relatively Na⁺-tolerant SC than in RL roots. In this report, tissue Na⁺ distribution was analyzed on a tissue dry weight basis and microscopically visualized using CoroNa-Red and CoroNa-Green dyes by laser scanning confocal microscopy. We also wanted to determine if fluorescence-based visualization of cellular Na⁺ distribution corresponded to tissue Na⁺ concentration expressed on a dry weight basis.

Materials and Methods

Plant material. Uniform 3-month-old seedlings of the salt-sensitive rootstock RL (*Citrus jambhiri* Lush.) and the relatively salt-tolerant SC [*C. paradisi* Macf. × *C. trifoliata* (L.) Raf] were purchased from a commercial nursery and transplanted into 0.5-L plastic pots

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filled with a peat, perlite, vermiculite (3:1:1) soilless media. Seedlings were grown in a greenhouse under natural photoperiods during the late summer when maximum photosynthetically active radiation at the plant level was $\approx 1200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (LI-170; LICOR, Lincoln, NE). The average day/night temperature was 36/21 °C and relative humidity varied diurnally from 40% to 100%. Plants were irrigated every other day with a dilute solution of a complete fertilizer (8N-0.7P-6.6K) at 100 mg·L⁻¹ nitrogen (N) plus 6% iron chelate in a sufficient volume to leach from the bottom of all pots. Plants received

≈ 21 mg of N per week. One month after transplanting, 50 mM NaCl (salinized) was gradually added to the nutrient solution of half of the plants. To avoid an osmotic shock, salinity was increased in increments of 10 mM NaCl per day until 50 mM NaCl was achieved. The experimental design was a two rootstock $\times$ two salinity level (0, 50 mM NaCl) with 12 replicates per treatment. Two replicate plants were harvested and analyzed periodically during the experimental period of 42 d; there were three replicate seedlings of each rootstock that were either salinized or not for 42 d.

All leaf measurements used fully expanded mature leaves from the midstem area on each seedling. As an index of leaf chlorophyll, leaf greenness was estimated using a SPAD meter (SPAD-502; Minolta Corp., Ramsey, NJ).

Near the end of the experiment, whole plant transpiration (E_p) was measured gravimetrically by daily weight loss from three replicate pots sealed in a plastic bag at the base of the stem, divided by the total leaf area at harvest, and expressed in units of $\text{g}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$. Harvested plants were separated into leaves, stems, and roots, briefly rinsed in deionized water, and oven-dried at 60 °C for at least 48 h. Dried mature leaves and fibrous roots were ground to a powder and leaf N, Na⁺, and Cl⁻ concentrations were determined in a commercial laboratory (Waters Agricultural Laboratory, Camilla, GA) and expressed as a percentage dry weight.

Confocal microscopy. At periodic harvest times, water rinsed roots of RL and SC were excised 0.5 cm from the root tip and leaves were separated at the base of the petiole. Root tissue was sliced into 1- to 2-mm segments and incubated in an incubation solution containing 20 mM Buffer MOPS pH 7.0, 0.5 mM CaSO₄, and 200 mM sorbitol (incubation media) to recover from effects of excision and sudden changes in osmolarity and salt concentration (Davenport and Tester, 2000). Sampled leaves were sliced in segments of approximately the same size as roots at the time of sampling.

For Na⁺ compartmentation analysis by fluorescent laser scanning confocal microscopy (Leica TCS SL; Leica, Heidelberg, Germany), leaf and root segments from 0 and 50 mM treated RL and SC citrus plants were incubated in 10 μM of cell permeant CoroNa-Red fluorescent sodium indicator (Molecular Probes, C-24431; Invitrogen, Eugene, OR) in the incubation media above for 12 h. Tissue segments were washed three times to remove any excess fluorescent dye using the incubation solution without the CoroNa-Red. The microscope setting for detecting CoroNa-Red was $\lambda_{\text{exc}} = 543$ nm and λ_{em} over the 565- to 600-nm spectral band. A series of confocal optical XY images through the thickness of the samples (total scanning volume was $\approx 150 \mu\text{m}$ with a slice thickness of 3 μm) were acquired in XYZ scanning mode using the Leica TCS SL software package. Comparison of different levels of fluorescence between cells was carried out by visualizing cells with the identical imaging settings of the confocal microscope (i.e., laser intensity, pinhole diameter, and settings of the imaging detectors).

Protoplast isolation and observation. Protoplasts from SC roots were isolated by incubating root segments overnight in a cell wall hydrolytic solution containing 50 mM BTP/MES buffer (pH 5.6), 800 mM sorbitol, 2% Cellulase Onozuka RS (from *Trichoderma viride*; SERVA Electrophoresis GmbH, Germany), 1.25% Macerozyme R-10 from *Rhizopus* sp. (SERVA Electrophoresis GmbH, Germany), 0.5% pectinase from *Aspergillus*

Fig. 1. Visual appearance of rough lemon (RL) and Swingle citrumelo (SC) plants after 42 d with and without salt treatment.

Fig. 2. Accumulation of Na⁺ and Cl⁻ in leaves and roots of Swingle citrumelo (SC) and rough lemon (RL) citrus rootstocks irrigated with 50 mM NaCl during 42 d. **Significant at $P < 0.01$. Each symbol represents one plant.

niger (CALBIOCHEM, Germany), 0.1% polyvinylpyrrolidone-40, 0.05% bovine serum albumin, and 1 mM CaCl₂. After 12 h of incubation, the protoplasts were washed with the hydrolytic solution without the enzymes and then incubated with 10 μM cell-permeant CoroNa-Green Sodium Indicator (Molecular Probes, C-36676; Invitrogen) for an additional 8 h. Fluorescent laser scanning images with CoroNa-Green were obtained using λ_{exc} = 488 nm and λ_{em} over the 510- to 540-nm spectral band. Some of the root protoplasts were incubated with 5 μM Hoechst 33342 (Molecular Probes, H3570; Invitrogen) together with CoroNa-Green and observed under fluorescent microscopy with corresponding green and blue fluorescence filters. Photographs were taken with a Canon digital camera adapted to the microscope.

Results

Plant growth and ion concentration. All non-salinized seedlings appeared healthy and showed no signs of impaired growth. Salinized RL seedlings appeared chlorotic and had visible tip burn on most leaves after 40 d of salt treatment (Fig. 1). Salinized SC plants grew less than control plants but did not display any visible phytotoxic symptoms. At the end of the experimental period, root Na⁺ concentrations in SC exceeded leaf Na⁺ concentrations but root and leaf Cl⁻ concentrations were similar (Fig. 2A–B). In SC, leaf Cl⁻ concentrations far exceeded levels considered to be toxic in bearing trees (0.7%; Obreza and Morgan, 2008). In RL, both Na⁺ and Cl⁻ concentrations in salinized leaves exceeded concentration in roots, and concentrations

of both ions in salinized leaves exceeded toxic levels after approximately Day 25 (Fig. 2C–D).

Leaf Na⁺ in salinized RL exceeded concentrations in salinized SC leaves (Table 1); leaf Cl⁻ levels in RL were numerically higher but not significantly different from SC leaves. Although all plants were well fertilized, the largest non-salinized RL seedlings developed low leaf N below optimum (Obreza and Morgan, 2008) but all other plants had leaf N concentrations at or above optimum values of 2.7%. Salinity reduced E_p and leaf chlorophyll in both rootstock types but E_p did not differ between rootstock types. RL leaves had lower chlorophyll indices than SC leaves; however, thus salinized RL had the lowest leaf chlorophyll values.

Tissue sodium compartmentation analysis. Na⁺ distribution at the tissue and cell levels in young roots and leaves was determined by CoroNa-Red fluorescence. CoroNa-Red is a water-soluble cell-permeant indicator, which fluoresces only after binding to Na⁺. In non-salinized control roots of both tissue types, Na⁺-induced fluorescence was observed primarily in cortical cells scattered throughout (Figs. 3A and 3C). SC roots accumulated higher levels of Na⁺ than those of RL. The small but noticeable fluorescence signal in the absence of added Na⁺ in Figures 3A and 3C likely resulted from the inherent presence of Na⁺ in irrigation water, soils, and roots. In the salinized treatment, however, a larger number of Na⁺ accumulating cells was observed in roots of SC compared with RL roots (Figs. 3B and 3D). The higher levels of Na⁺ accumulation in SC roots were consistent throughout all samples collected and through the length of the root tips (Fig. 4). In some instances, parenchyma cells within the stele also fluoresced indicating their high Na⁺ content (arrowhead in Fig. 3B).

Contrary to roots, compartmentation analysis of Na⁺ distribution in leaves from salinized trees demonstrated that RL accumulated considerably larger amounts of Na⁺ in mesophyll parenchyma cells than those of SC (Fig. 5). The large difference in Na⁺ accumulation was consistent in both lamina tissue as well as in the petiole, where accumulation occurred both in cortical cells as well as in pith parenchyma (Figs. 5B and 5D).

Intracellular sodium compartmentation analysis. Fluorescent micrographs (Fig. 6A) and three-dimensional reconstruction analysis of root cortical cells (Fig. 6B–C) confirmed the vacuole as the main storage compartment for cellular Na⁺. Figure 6B shows the three-dimensional topographic outline of several vacuoles of root cells where concave areas represent the location of organelles and cytosolic areas. A prominent feature of all cells was their fluorescent walls and the presence of a brighter cytosolic zone (arrow in Fig. 6B–C) indicating an area where Na⁺ concentration is considerably higher than in the central vacuole. The higher fluorescence intensity of this zone also was apparent in the topographic three-dimensional analysis in Figure 6C.

Table 1. Effects of rough lemon (RL) and Swingle citrumelo (SC) rootstock and 42 d of salt treatment (0 or 50 mM NaCl) on mean (n = 3) plant transpiration (E_p), chlorophyll (SPAD) index, leaf nitrogen, sodium and chlorine concentrations of 6-month-old seedlings.²

Rootstock	Salt	E _p	Chlorophyll index	Leaf nitrogen	Chlorine	Sodium
		(g·m ⁻² ·h ⁻¹)		(% dry wt)		
RL	0 mM	65 a	49 b	2.3 c	0.2 b	0.2 bc
RL	50 mM	25 b	23 c	2.7 b	2.9 a	2.3 a
SC	0 mM	59 a	62 a	3.1 a	0.2 b	0.1 c
SC	50 mM	38 b	50 b	3.1 a	2.2 a	0.5 b

²Values within a column followed by different letters differ significantly at *P* < 0.05.

Fig. 3. Laser scanning confocal images of roots from Swingle citrumelo (SC) and rough lemon (RL) without NaCl treatment (A, C) and after 50 mM NaCl treatment (B, D) for 42 d. Root segments were cut perpendicularly and incubated in CoroNa-Red added before visualization. Arrow in B denotes Na⁺ accumulating cell in the pericycle.

Further observations of the brightly fluorescent structure were carried out using CoroNa-Green with its higher fluorescence intensity compared with CoroNa-Red. To avoid possible interference with the cell walls, protoplasts were generated after tissue incubation in Na^+ . At higher magnification, two types of protoplasts were observed. A set of

protoplasts contained a single brightly fluorescent CoroNa-Green zone (Fig. 7A), whereas others also exhibited fluorescence in the vacuole (Fig. 7C). The bright CoroNa-Green zone remained visible even when the vacuole showed fluorescence (Fig. 7C), denoting a higher Na^+ concentration. Despite exhibiting higher fluorescent intensity and therefore

higher Na^+ concentration, is very likely that the vacuole, with its much larger size, was capable of storing large amounts of Na^+ . Fluorescent micrographs using both CoroNa-Green and Hoechst 33342, a nucleic acid-specific stain (Fig. 8), demonstrated that this bright structure did not represent the nucleus. The clear separation of blue and green fluorescence areas indicates that these two structures represent separate entities corresponding to the nucleus (blue) and to an unidentified high Na^+ -containing compartment of ≈ 2 to $6\ \mu\text{m}$ in diameter.

Confocal images of salinized root segments washed with 1 M KCl for 2 h revealed the unexpected persistence of Na^+ in the cell wall space (Fig. 9). Control tissue incubated in the absence of CoroNa-Red displayed no autofluorescence (Fig. 9A–B). When salinized tissue was washed in incubation media for 2 h and then incubated in CoroNa-Red, intense fluorescence remained throughout (Fig. 9C–D). However, when salinized root segments were washed with 1 M KCl for 2 h before incubation with CoroNa-Red, fluorescence remained only within the stele. Very little fluorescence, if any, persisted in the cortical wall spaces, although some fluorescence remained along the epidermis (arrowhead in Fig. 9F) and casparian band (arrow in Fig. 9F).

Discussion

Plant growth and leaf ion concentration (Figs. 1 and 2; Table 1) confirmed the previously described relatively high Cl^- sensitivity of RL and SC (Levy and Syvertsen, 2004). In addition, concentrations of Na^+ in salinized RL leaves were four times greater than in SC leaves supporting previously described rootstock difference in Na^+ accumulation. Na^+ sequestration in root organs has been reported in a variety of salt-tolerant species (Apse and Blumwald, 2007; Blumwald et al., 2000; Cuin et al., 2011; Möller et al., 2009) and also in citrus rootstocks differing in salt tolerance (Garcia-Sanchez and Syvertsen, 2006). Although the ability to sequester Na^+ ions in roots, thereby preventing toxic levels of Na^+ from reaching leaf tissues, has been previously reported in citrus, this is the first report describing cellular and tissue distribution of Na^+ together in root and leaf tissues of citrus rootstocks differing in their Na^+ tolerance.

Sequestration of Na^+ away from the cytosol of photosynthetic cells was accomplished in a variety of ways both at the tissue and cell structural levels. Although both citrus varieties appeared to have similar compartmentation strategies at the cell level, our data demonstrated that retention of Na^+ in the roots provided better protection against Na^+ toxicity than cell compartmentation in leaves (Figs. 3 and 5). This conclusion is based on two pieces of evidence. First, tissue Na^+ analysis showed a 2-fold higher Na^+ concentration in salinized roots of Na^+ -tolerant SC than in RL (Fig. 2). Second, fluorescence analysis of Na^+ localization within the tissue

Fig. 4. Laser scanning confocal images of Swingle citrumelo (SC) roots after 50 mM NaCl treatment for 42 d. Roots were cut longitudinally incubated in CoroNa-Red before observation (A). B is a close-up showing the cortical nature of the accumulating cells.

Fig. 5. Laser scanning confocal images of leaves from Swingle citrumelo (SC) and rough lemon (RL) without NaCl treatment (A, C) and after 50 mM NaCl treatment (B, D) for 42 d. Leaf segments were cut transversely and labeled with CoroNa-Red before visualization.

Fig. 6. Three-dimensional images (A–B) of Swingle citrangelo (SC) roots supplied with 50 mM NaCl for 42 d. (A) Na⁺ accumulation is evident in cortical cells and in cells inside the pericycle. (B) Three-dimensional image of vacuoles within the cortical region. Arrow points to the highly fluorescent structure commonly observed. (C) Topographic three-dimensional analysis of B showing the intensely fluorescent sodium (Na)-accumulating structures (arrows) peripheral to the larger vacuole.

indicated higher fluorescence in SC root cortical cells than in RL roots (Fig. 3). In addition, fluorescence-based visualization

of cellular Na⁺ distribution corresponded to tissue Na⁺ concentration expressed on a dry weight basis.

Retention of Na⁺ within the roots has been proposed as a key mechanism providing Na⁺ tolerance to glycophytes (Conn and Gilliham, 2010). Our observations support that idea in that the epidermal and cortical cells accumulated symplastic Na⁺ before reaching the vasculature. In citrus, however, Na⁺ was predominantly accumulated in root cortical cells unlike *Arabidopsis* wild-type (Oh et al., 2009), where substantial Na⁺ was retained by epidermal cells. The pericycle also accumulated Na⁺ in SC roots (Fig. 3B). This may be a critical mechanism in the relatively high Na⁺ tolerance in SC because the pericycle remains the last metabolic barrier before solutes enter the xylem stream to move toward leaves (Conn and Gilliham, 2010).

Within leaves, there appeared to be no discriminatory compartmentation between cell types because Na⁺ appeared to be accumulated in photosynthetic cells as well as in parenchyma cells (Fig. 5). Thus, the difference between the Na⁺ accumulation in SC and RL could be attributed to the greater amount of Na⁺ that bypassed the roots and was allowed to reach the leaves in RL.

Fluorescent scanning micrographs (Figs. 3 to 7) verified the vacuole as the largest Na⁺ depository in both root and leaf tissues where the vacuole displayed its higher Na⁺ content compared with the cytosol. As a storage compartment, the vacuolar membrane contains a variety of Na⁺-related transporters capable of removing toxic levels of Na⁺ from the cytosol. Aside from Na⁺ channels, the vacuole contains a Na⁺/H⁺ antiporter (Apse and Blumwald, 2007). The relevance of this tonoplast-bound Na⁺ transporter to salt tolerance has been thoroughly demonstrated in several species such as tomato (Zhang and Blumwald, 2001), *Arabidopsis* (Shi et al., 2003), sugarbeet (Liu et al., 2008), and cotton (He et al., 2005), where increasing expression of the Na⁺/H⁺ antiporter resulted in increased salt tolerance. It is noteworthy that not all citrus cells in a given tissue exhibited similar fluorescence intensity, and in some instances, there was no visible fluorescence. This is not uncommon because cryo-scanning electron microscopy (Läuchli et al., 2008; Möller et al., 2009) and radiographic microanalysis (Flowers and Hajibagheri, 2001) of root cells from different species demonstrated that adjacent cells can have different vacuolar Na⁺ concentrations.

Apart from the vacuole, most cells also contained organelle-like, 2- to 6-μm structures (Figs. 6 to 8) that exhibited higher fluorescence intensity. We cannot identify the nature of these structures based on our analyses; however, when these structures were viewed through successive Z planes, it became evident that this was a membrane-bound cytosolic organelle-like structure. In many cells, this was the only fluorescent structure, and all cells that displayed vacuolar fluorescence also contained this organelle-like structure (Fig. 6). It is noteworthy that, although vacuolar sequestration of Na⁺ was minimal in RL roots, most cells displayed this highly fluorescent structure (Fig. 3C–D).

Fig. 7. (A–C) Root protoplast from Swingle citrumelo (SC) grown in 50 mM NaCl for 42 d. The figure is a composite of laser scanning confocal microscopy with and without fluorescence. The highly fluorescent cellular structure labeled with CoroNa-Green can be observed against the opaque cell in A, whereas in C, the highly fluorescent structure outshines the Na⁺-accumulating vacuole.

From these observations, we conclude that Na⁺ was initially sequestered in this type organelle-like structure followed by Na⁺ accumulation in the vacuole. The significance of this structure is uncertain, but it appears that Na⁺ could be stored in two intracellular compartments. Similar observations of a highly intense Na⁺-containing organelle in *Arabidopsis* cortical cells at the root tip were made by Hamaji et al. (2009). Although speculative at this point, it is likely that this membrane-bound structure represents an intermediate stage or a delivery point for Na⁺ in the process of endocytosis as in *Arabidopsis* root cells (Oh et al., 2009). This process has been suggested to be involved in ion uptake into plant cells (MacRobbie, 1971). It is also possible that these small vesicles accumulate Na⁺ and then merge with the main vacuole as observed in yeast cells (Nass and Rao, 1998).

It is generally assumed that once ions pass the endodermis in roots, ions move unrestricted following the movement of water to the xylem vessels (Peng et al., 2004). Extrusion of excess Na⁺ from the cytosol of parenchyma cells by plasma membrane-bound Na⁺ antiporters (Shi et al., 2000) within the epidermis and stele would be impractical if a trapping system were not present to prevent Na⁺ from entering the adjacent xylem

vessels. A prominent feature in our fluorescent micrographs of all tissues incubated in CoroNa-Red was the intense fluorescence of the cell walls, especially in the thick-walled xylem and proto-xylem elements (Figs. 3 to 6 and 9). The intense fluorescence was evident in roots from both non-salinized control and NaCl-treated plants and was not the result of autofluorescence because roots incubated without CoroNa-Red lacked the fluorescent red signal (Fig. 9A–B). The apoplast is generally considered as a cation-binding matrix because it contains many disassociated carboxyl residues found in the pectic portion of the cell walls (Plett and Möller, 2010). Because the 2-h K⁺ wash only removed Na⁺ from cortical cell walls but not from the stele (Fig. 9E–F), it is reasonable to assume that the cell walls of the xylem and surrounding stele tissue are capable of retaining Na⁺ providing an additional Na⁺ retention barrier. Na⁺ adsorption by root cell walls has been previously demonstrated in barley roots (Stassart et al., 1981) and for metals in a wide range of plants (Mari and Lebrun, 2006). It is therefore possible that the cell walls of the stele could provide an auxiliary structural trapping process, regardless of the cellular compartmentation capabilities.

Our results support our original hypothesis that Na⁺ sequestration in the root vacuoles

Fig. 8. (A) Light micrograph image of a protoplast from Swingle citrumelo (SC) roots grown in 50 mM NaCl and incubated in Hoechst 33342 and CoroNa-Green before observation. (B) Fluorescent micrograph of A.

of the relatively Na⁺-tolerant SC would be greater than in RL roots. Vacuolar sequestration, therefore, is a significant contributing mechanism to the ability of SC roots to maintain lower levels of Na⁺ in leaves compared with leaves associated with RL roots. This adds to our understanding of mechanisms of salinity tolerance in contrasting citrus rootstocks. Distribution of fluorescence in SC root protoplasts verified vacuolar localization of Na⁺ and tissue concentrations expressed on a dry weight basis.

Literature Cited

- Apse, M.P. and E. Blumwald. 2007. Na⁺ transport in plants. *FEBS Lett.* 581:2247–2254.
- Blumwald, E., G.S. Aharon, and M.P. Apse. 2000. Sodium transport in plant cells. *Biochim. Biophys. Acta* 1465:140–151.
- Castle, W.S., K. Bowman, J.H. Graham, and D.P.H. Tucker. 2006. Florida citrus rootstock selection guide. UF/IFAS, SP-248. Univ. FL Ext. Serv., Gainesville, FL.
- Castle, W.S., D.P.H. Tucker, A.H. Krezdorn, and C.O. Youtsey. 1993. Rootstocks for Florida citrus. 2nd Ed. UF/IFAS SP-42. Univ. FL Ext. Serv., Gainesville, FL.
- Conn, S. and M. Gilliham. 2010. Comparative physiology of elemental distributions in plants. *Ann. Bot. (Lond.)* 105:1081–1102.
- Cuin, T.A., J. Bose, G. Stefano, D. Jha, M. Tester, S. Mancuso, and S. Shabala. 2011. Assessing the role of root plasma membrane and tonoplast Na⁺/H⁺ exchangers in salinity tolerance in wheat: *In planta* quantification methods. *Plant Cell Environ.* 34:947–961.
- Davenport, R.J. and M. Tester. 2000. A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. *Plant Physiol.* 122:823–834.

Fig. 9. Light micrographs and laser scanning confocal images of roots segments from Swingle citrumelo (SC) treated with 50 mM NaCl for 42 d. (A) Light micrograph of control roots without added CoroNa-Red sodium indicator. (B) Laser scanning confocal image of A. (C) Light micrograph of control roots incubated in CoroNa-Red and washed with a 10 mM buffer solution. (D) Laser fluorescent scanning confocal image of C. (E) Light micrograph of control roots incubated in CoroNa-Red and washed with a 1 M KCl for 2 h. (F) Laser scanning confocal image of E. (Arrow indicates the casparian strip, whereas the triangular arrowhead denotes the epidermis.)

Flowers, T.J. and M.A. Hajibagheri. 2001. Salinity tolerance in *Hordeum vulgare*: Ion concentrations in root cells of cultivars differing in salt tolerance. *Plant Soil* 231:1–9.

Garcia-Sanchez, F.G. and J.P. Syvertsen. 2006. Salinity tolerance of Cleopatra mandarin and Carrizo citrange citrus rootstock seedling is affected by CO₂ enrichment during growth. *J. Amer. Soc. Hort. Sci.* 131:24–31.

Garcia-Sanchez, F.G. and J.P. Syvertsen. 2009. Substrate type and salinity affect growth allocation, tissue ion concentrations and physiological responses of Carrizo citrange seedlings. *HortScience* 44:1432–1437.

Hamaji, K., M. Nagira, K. Yoshida, M. Ohnishi, Y. Oda, T. Uemura, T. Goh, M.H. Sato, M.T. Morita, M. Tasaka, S. Hasezawa, A. Nakano, I. Hara-Nishimura, M. Maeshima, H. Fukaki, and T. Mimura. 2009. Dynamic aspects of ion accumulation by vesicle traffic under salt stress in *Arabidopsis*. *Plant Cell Physiol.* 50:2023–2033.

Hasegawa, P.M., R.A. Bressan, J.K. Zhu, and H.J. Bohnert. 2000. Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Mol. Biol.* 51:895–901.

He, C., J. Yan, G. Shen, L. Fu, S. Holaday, D. Auld, E. Blumwald, and H. Zhang. 2005. Expression

of an *Arabidopsis* vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. *Plant Cell Physiol.* 46:1848–1854.

Läuchli, A., R.A. James, C.X. Huang, M. McCully, and R. Munns. 2008. Cell-specific localization of Na⁺ in roots of durum wheat and possible control points for salt exclusion. *Plant Cell Environ.* 31:1565–1574.

Levy, Y. and J.P. Syvertsen. 2004. Irrigation water quality and salinity effects in citrus trees. *Hort. Rev.* 30:37–82.

Liu, H., Q. Wang, M. Yu, Y. Zhang, Y. Wu, and H. Zhang. 2008. Transgenic salt-tolerant sugar beet (*Beta vulgaris* L.) constitutively expressing an *Arabidopsis thaliana* vacuolar Na⁺/H⁺ antiporter gene, *AtNHX3*, accumulates more soluble sugar but less salt in storage roots. *Plant Cell Environ.* 31:1325–1334.

Maas, E.V. 1993. Salinity and citriculture. *Tree Physiol.* 12:195–216.

MacRobbie, E.A.C. 1971. Fluxes and compartmentation in plant cells. *Annu. Rev. Plant Physiol.* 22:75–96.

Mari, S. and M. Lebrun. 2006. Metal immobilization: Where and how? *Topics Cur. Gene* 14:273–298.

Møller, I.S., M. Gilliam, D. Jha, G.M. Mayo, S.J. Roy, J.C. Coates, J. Haseloff, and M. Tester. 2009. Shoot Na⁺ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na⁺ transport in *Arabidopsis*. *Plant Cell* 21:2163–2178.

Munns, R. and M. Tester. 2008. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59: 651–681.

Nass, R. and R. Rao. 1998. Novel localization of a Na⁺/H⁺ exchanger in a late endosomal compartment of yeast. *J. Biol. Chem.* 273: 21054–21060.

Obreza, T.A. and K.T. Morgan. 2008. Nutrition of Florida citrus trees. UF/IFAS SL 253. Univ. FL, Gainesville, FL.

Oh, D.-H., E. Leidi, Q. Zhang, S.-M. Hwang, Y. Li, F.J. Quintero, X. Jiang, M.P. D'Urzo, S.Y. Lee, Y. Zhao, J.D. Bakh, R.A. Bressan, D.-J. Yun, J.M. Pardo, and H.J. Bohnert. 2009. Loss of halophytism by interference with SOS1 expression. *Plant Physiol.* 151:210–222.

Peng, Y.H., Y.F. Zhu, Y.Q. Mao, S.M. Wang, W.A. Su, and Z.C. Tang. 2004. Alkali grass resists salt stress through high [K⁺] and endodermis barrier to Na⁺. *J. Expt. Bot.* 55:939–949.

Plett, D.C. and I.S. Møller. 2010. Na⁺ transport in glycophytic plants: What we know and would like to know. *Plant Cell Environ.* 33:612–626.

Redjala, T., T. Sterckeman, S. Skiker, and G. Echevarria. 2010. Contribution of apoplast and symplast to short-term nickel uptake by maize and *Leptoplax emarginata* roots. *Environ. Exp. Bot.* 68:99–106.

Shi, H., M. Ishitani, C. Kim, and J.K. Zhu. 2000. The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na⁺/H⁺ antiporter. *Proc. Natl. Acad. Sci. USA* 97:6896–6901.

Shi, H., B.H. Lee, S.J. Wu, and J.K. Zhu. 2003. Overexpression of a plasma membrane Na⁺/H⁺ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat. Biotechnol.* 21:81–85.

Shi, H., F.J. Quintero, J.M. Pardo, and J.K. Zhu. 2002. The putative plasma membrane Na⁺/H⁺ antiporter SOS1 controls long-distance Na⁺ transport in plants. *Plant Cell* 14:465–477.

Stassart, J.M., L. Neirinckx, and R. Dejaegre. 1981. The interactions between monovalent cations and calcium during their adsorption

- on isolated cell walls and absorption by intact barley roots. *Ann. Bot. (Lond.)* 47:647–652.
- Syvertsen, J.P., J.C. Melgar, and F. Garcia-Sanchez. 2010. Salinity tolerance and leaf water use efficiency in citrus. *J. Amer. Soc. Hort. Sci.* 135:33–39.
- Tester, M. and R. Davenport. 2003. Na⁺ tolerance and Na⁺ transport in higher plants. *Ann. Bot. (Lond.)* 91:503–527.
- Zhang, H.-X. and E. Blumwald. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnol.* 19:765–768.
- Zhang, H.-X., J.N. Hodson, J.P. Williams, and E. Blumwald. 2001. Engineering salt-tolerant *Brassica* plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA* 98:12832–12836.
- Zhu, J.-K. 2000. Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.* 124: 941–948.
- Zekri, M. and L.R. Parsons. 1992. Salinity tolerance of citrus rootstocks: Effects of salt on root and leaf mineral concentrations. *Plant Soil* 147:171–181.