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Pathways of cadmium fluxes in the root of the halophyte *Suaeda salsa*

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ABSTRACT

Halophyte plants offer a greater potential for phytoremediation research for reducing the levels of toxic metals from saline soils than salt sensitive plants. Using the scanning ion-selective electrode technique, we analyzed the pattern and rate of Cd^{2+} fluxes at different regions of the root apex of *Suaeda salsa*. The Cd^{2+} influx in the rhizosphere was greatest near the root tip (within 150 μm of the tip). The results indicated that Cd^{2+} influx into roots was significantly suppressed by the pre-treatment or in the presence of two kinds of Ca^{2+} channel blockers; $LaCl_3$ and verapamil. The Cd^{2+} influx was also reduced by *N*-ethylmaleimide, a thiol blocker. Cd content determination and labeling of Cd using fluorescent dye support our conclusion. The results of this study provide a more stable theoretical basis for the phytoremediation of Cd contamination in saline soils of coastal zones.

1. Introduction

Cadmium (Cd) is a widespread heavy metal in the environment largely because of intensive anthropogenic activities, including industrial, agricultural and/or urban development. Cd contamination still increases in coastal regions and their neighboring estuaries of China, to a large extent as a result of runoff (Zho et al., 2004). Therefore, remediation of Cd polluted soils is required and the use of plants (phytoremediation) which can absorb and sequester large quantities of this toxic metal has been suggested (Moffat, 1995). Salt-adapted plants, halophytes, are able to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more (Flowers and Colmer, 2008). These plants have been suggested to be better adapted to cope with environmental stresses, such as heavy metals compared to salt-sensitive plants commonly chosen for phytoremediation purposes. Halophytes thus offer a greater potential for phyto-remediation research for reducing the levels of toxic metals from saline soils than commonly studied plants (Manousaki and Kalogerakis, 2011). The species that was selected for this study, *Suaeda salsa*, is inhabited in Liaodong Bay, located in the northwest of the Bohai Sea. Several large heavily polluted rivers including the Liaohe River, one of the most heavily polluted rivers in China, drain into the Liaodong Bay. As a result, Liaodong Bay has become a heavily polluted site due to the excessive discharge of heavy metals (Xu et al., 2009). *S. salsa* has a high capacity of heavy metal accumulation (Zhu et al., 2005), hence indicating its potential in phyto-remediation of heavy metal contaminated soil. A fundamental understanding of the Cd^{2+} uptake mechanisms in the roots of *S. salsa* would be a critical issue for its application in the phyto-remediation of metal contaminated sites of the coastal zone. The characteristics of metal transport in the membrane of different plants may not be identical between halophytes and non-halophytes, although some hypotheses have been formulated to explain this process for the latter group of plants. As a non-essential element for plants, cadmium has been assumed to be taken up by transporters for essential elements as a consequence of lack of specificity of the transporters (Pence et al., 2000; Cohen et al., 1998; Connolly et al., 2002; Besson-Bard et al., 2009).

To identify possible pathways for Cd^{2+} uptake, to date, most reports have dealt with the analysis of the overall changes in ion content in plant tissues or with monitoring the kinetics of nutrient depletion in a growth solution (Tripathi et al., 1995). Such approaches integrate ion uptake over the entire tissue surface, providing an averaged measurement over a period of time. Due to methodological limitations (relatively poor spatial and temporal resolution), these experiments failed to provide answers about the specific ionic uptake mechanisms involved. The scanning ion-selective electrode technique (SIET) or microelectrode ion flux measurement (MIFE) technique has

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given us a new opportunity to successfully address the issues raised above (Newman, 2001; Newman et al., 1987; Shabala, 2003, 2006; Kunkel et al., 2001, 2005; Xu et al., 2006). The technique now allows us to measure specific ion fluxes from different regions of the root under practical physiological conditions (Newman, 2001; Pineros et al., 1998).

Although Cd entry to root cells is the first key process for phytoremediation, only several reports have currently described the dynamics of Cd^{2+} flux along root surface by employment of ion-selective microelectrodes (He et al., 2011; Farrell et al., 2005; Pineros et al., 1998). Up to now, little is known about the dynamics of Cd^{2+} flux in the rhizosphere of halophytes plants. In this study, the SIET was used to investigate the spatial and temporal characteristics of the transmembrane Cd^{2+} fluxes at the roots apex of *S. salsa*. Also, to test if Ca^{2+} channel and SH-binding ligands were involved in Cd transport by *S. salsa*, the net Cd^{2+} fluxes across the root were monitored after pre-treatment or in the presence of a metabolic inhibitor (*N*-ethylmaleimide) and of two Ca^{2+} channel blockers (verapamil, lanthanum as $LaCl_3$).

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *S. salsa* were collected from the Yellow River Delta in Shandong Province, PR China. Prior to germination, the seeds were surface sterilized in 0.5% NaOCl for 15 min. The seeds were then germinated in the dark on moist filter paper with deionized water. After 2 to 3 days, germinated seeds were transferred to Nylon mesh suspended over growing solution containing 1.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.1 mM NaCl and 1.0 mM KNO_3 at pH 6.5 in 1 L HDPE containers. Seedlings were grown in a growth chamber at 25/15 °C (light:dark, 16:8 h) under a light intensity of 300 μmol photons $m^{-2}\,s^{-1}$. Plants were grown for 1 week before being used for the flux studies. The length of the whole root system was about 5–10 cm.

2.2. Net Cd^{2+} fluxes measurement

The net Cd^{2+} flux in the rhizosphere was measured noninvasively using a Cd selective microelectrode and scanning ion-selective electrode technique (SIET system BIO-001A; Younger USA, LLC, MA, USA). The reference electrode consisted of an Ag/AgCl wire in a glass micropipette (tip diameter ca. 50–100 μm) containing 0.5 M KCl in a 1% agar solution. The Cd ion-selective microelectrodes with an external tip diameter of approximately 3 μm were manufactured and silanized with tributylchlorosilane and the tips backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909, Fluka, Buchs, Switzerland). The microelectrodes were calibrated in 50, 100 and 500 μM Cd^{2+} prior to the Cd^{2+} flux measurement. Only electrodes with Nernstian slopes >25 mV per decade were used. Details on fabrication and calibration of Cd^{2+} ion selective microelectrodes have been described previously (Ma et al., 2010).

With the plant intact, the primary (longest) root was mounted horizontally in the measuring chamber, and loosely fixed in place with dental wax. After mounting the plant in the chamber, the dish was placed on an inverted microscope in a Faraday cage and filled with 5 mL of measuring solution consisting of 100 μM $Cd(NO_3)_2$ and 20 μM KCl (solution pH=6.5). Whereas the shoot was kept out of the bath solution, all the roots were immersed in the bath solution but were kept away from the primary root. The reference electrode tip was placed in the bath solution and kept at a distance of at least 1 cm from any of the roots. Gradients of Cd^{2+} adjacent to the root were measured by moving the Cd^{2+}-selective microelectrode using a computer-controlled stepper motor between two positions in a pre-set excursion of 20 μm.

The flux data were obtained with the ASET software, which is part of the SIET system. Eventually, the raw data from all the measurements, including background-mV estimation of concentration and the microvolt difference estimation of the local gradient, were converted into net Cd fluxes (pmol $cm^{-2}\,s^{-1}$) using MageFlux, developed by the Xu-Yue company (http://xuyue.net/mageflux).

2.3. Pre-treatment with a metabolic inhibitor and ion channel blockers

In order to elucidate the transporter(s) responsible for mediating the Cd^{2+} influx, a series of pharmacological experiments were carried out on *S. salsa*. Three pharmacological agents were chosen for this experiment. Verapamil (a known Ca^{2+} channel blocker, Andrejauskas et al., 1985), $LaCl_3$ (a nonselective cation channel current (NSCC) blocker) and *N*-ethylmaleimide, NEM (–SH inhibitor) were used to modify the activity of selected plasma membrane transporters. All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. These inhibitors were mixed with the basic solution (1.0 mM Ca $(NO_3)_2$, 0.5 mM $MgSO_4$, 0.1 mM NaCl and 1.0 mM KNO_3) to achieve their final concentrations that were as follows: Verapamil, 20 μM; $LaCl_3$, 50 μM; NEM, 10 μM. All these concentrations were selected based on previous literature reports showing that these concentrations are physiologically relevant (Wang and Fisher, 1999). Solution pH was adjusted to 6.5 (using HCl/NaOH) in all treatments. The control treatment was pre-exposed in pharmacological free medium. The plants were pre-exposed in solutions containing the pharmacological agents for 24 h prior to the measurement of the Cd fluxes and the uptake experiment. After pre-exposure, the plants were rinsed with de-ionized water and then transferred to test solutions to perform the measurements of the Cd^{2+} fluxes.

2.4. Transient experiments with metabolic inhibitor and ion channel blockers

To further verify possible effects of the pharmacological agents, the Cd^{2+} fluxes in the rhizosphere were monitored before and after the application of the pharmacological agents. In transient experiments, pharmacological agents were added after the root was transferred to the measuring chamber. As steady-state fluxes were reached and measured for 5 min, 5 mL of measuring solution containing a double concentration of an appropriate chemical was carefully added into the chamber, and the measurement continued for a further 5 min. Solution pH was adjusted to 6.5 in advance using NaOH/HCl, and no substantial changes in Cd^{2+} activity were caused by addition of any pharmaceutical. About 20 min is required for unstirred layer conditions to be reached. This period of time was discarded from the analysis and appears as a gap in the figures.

2.5. Cd analysis in root

After the Cd^{2+} flux measurement, the plants were transferred to solution containing 100 μM Cd and exposed continually for 24 h. Subsequently, plants were harvested, washed in 1.0 mM EDTA for 5 min, rinsed with deionized water, and weighed. The samples of each treatment were digested using a microwave digestion system (MAR-5, CEM Corporation, Matthews, NC, USA). Reagents (5 mL conc. HNO_3) were added, the vessels were closed, and the samples were heated in the microwave oven (program: heating at 15 min to 200 °C and holding at 200 °C for 15 min). After the program was completed and the vessels were cooled down, the digest was transferred to a 50 mL volume flask, made up to volume and filtered. Cd concentrations in the digest were determined by ICP-MS (Agilent 7500i, Agilent Technologies Co. Ltd, USA). GBW07605 tea leaves (State Bureau of Technical Supervision, People's Republic of China) were employed as certified reference materials for plant analyses. Measured concentrations did not deviate more than 7% from the reported certified concentrations for Cd.

2.6. Fluorescence labeling of Cd in the root apex

The Cd Probe Leadmium Green AM dye (MolecularProbes, Invitrogen, Calsbad, CA, USA) was used to investigate the distribution of Cd in roots of plants pre-treated with 100 μM Cd for 24 h. A stock solution of Leadmium Green AM was made by adding 50 μL of DMSO to one vial of the dye. This stock solution was then diluted with 1:10 of 0.85% NaCl. Roots were immersed in this solution for 60 min in the dark. Samples were observed using a confocal laser scanning microscope (Olympus FV-1000, Tokyo, Japan) with excitation at 488 nm and emission at 500–550 nm, and serial confocal optical sections were taken. Images were analyzed using the Olympus Fluoview viewer software (ver. 2.1.c, Olympus, Tokyo, Japan). All the images were taken at 50 magnification. Each test was repeated at least three times for each root hair developmental period.

3. Results

3.1. Localization of Cd^{2+} fluxes along the root apex of Suaeda salsa

Ion fluxes were mapped along root hairs using a Cd selective microelectrode and scanning ion-selective electrode technique. It was expected that functionally different root zones of the halophytes plants *S. salsa* would exhibit different Cd^{2+} flux responses. To test this hypothesis, the transient Cd^{2+} flux was measured in different regions along the root axis (approximately 30 μm increments) after exposure to the measuring solution. As can be seen from Fig. 1, the Cd^{2+} flux profile showed a clear spatial organization. Detailed mapping of the Cd^{2+} fluxes around the root tip indicated that a significantly high Cd^{2+} influx was localized at positions 100–200 μm from the root apex, with a steadily decreasing influx with distance away from this site (Fig. 1). Moreover, the flux was negligible at the very close apex