J. Plant Biol. (2009) 52:298–302
DOI 10.1007/s12374-009-9036-8

ORIGINAL RESEARCH

In Planta Measurements of Na^+ Using Fluorescent Dye CoroNa Green

Mehea Park · Hyosuk Lee · Jung-Sook Lee · Myung-Ok Byun · Beom-Gi Kim

Received: 19 January 2009 / Revised: 6 April 2009 / Accepted: 10 April 2009 / Published online: 16 June 2009

Abstract Fluorescent indicators of Na^+ are valuable tools for nondestructive monitoring of its spatial and temporal distribution in plants. We tested whether CoroNa Green fluorescent dye, a newly developed sodium indicator, is suitable for measuring relative concentrations *in planta.* To determine the ideal conditions for its use, we incubated NaCl-pretreated *Arabidopsis thaliana* seedlings with different concentrations of CoroNa Green and visualized fluorescence in each organ with a fluorescein isothiocyanate filter. When 50 μM of dye was applied, fluorescence was distributed more uniformly and intensely in the root tips than in other tissues. Under those conditions, fluorescence gradually increased in the root tips when Na^+ was bound to CoroNa Green for concentrations up to 100 mM NaCl. Confocal fluorescence microscopy revealed that when *Arabidopsis* seedlings were incubated with the same concentration of NaCl, the *sos1* mutant had much stronger fluorescence than the wild type. This report is the first to describe the properties of CoroNa Green for measuring Na^+ content in intact plants and demonstrates the usefulness of this technique for investigating the mechanism of Na^+ homeostasis.

Keywords Confocal microscope · CoroNa Green · Fluorescein isothiocyanate (FITC) · Sodium indicator

M. Park · H. Lee · J.-S. Lee · M.-O. Byun · B.-G. Kim (✉)
Bio-crops Development Division, National Academy of Agricultural Science, Rural Development Administration, Seodun-dong Suwon, Kyunggi-do 441-707, Republic of Korea
e-mail: bgkimpeace@rda.go.kr

M. Park
Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong 369-873, Republic of Korea

Although sodium is one of the most abundant cations on earth, it is nonessential and can even be toxic to plant cellular functions. Excessive Na^+ in the soil is one of the major limiting factors that adversely affect plant growth and reduce crop productivity (Munns 2002). Hence, an important issue for breeders and plant scientists is the development of salt-resistant crops and the identification of molecular and physiological mechanisms for Na^+ resistance.

Na^+-induced ion toxicity is the primary effect of salt stress in plants but can be reduced by excluding and/or sequestering sodium. During this exclusion, Na^+ export to extracellular spaces is controlled by plasma membrane Na^+/H^+ antiporters such as SOS1 (Wu et al. 1996; Munns 2002; Cheong and Yun 2007; Park et al. 2008). In sequestration, Na^+ storage in vacuoles is accomplished by vacuolar Na^+/H^+ antiporters, e.g., NHX1, that use energy derived from the proton electrochemical gradient across vacuolar membranes (Apse et al. 1999; Duan et al. 2007). These well-known mechanisms have been exploited to develop Na^+-resistant crops (Zhang et al. 2001; Shi et al. 2003). However, it remains unclear how soil Na^+ is taken up by the roots and transported to distal tissues. The homeostasis mechanism is being investigated in halophytes that exhibit extremely high resistance to Na^+ stress, with the aim of adapting that process toward the development of resistant glycophytes, which would include most crops.

To investigate the mechanism(s) in cells, tissues, and whole plants, technologies are indispensable for quantifying ion contents and dynamics in live cells. Methodologies such as inductively coupled plasma mass and AAA are being used to measure Na^+ contents in plants. However, they are destructive techniques and do not show ion dynamics in live cells. One method that has been used successfully to quantify ion content and show dynamics in live cells is ion-binding fluorescent dye analysis. In animal

systems, Na^+, Ca^{2+}, and K^+ ions in live cells have been detected with such dyes, including SBFI, PBFI, and fura-2, respectively. These dyes are also appropriate for plant cells (Negulescu and Machen 1990; Halperin and Lynch 2003; Kader and Lindberg 2005). Under saline conditions, SBFI has been used to examine Na^+ uptake and Na^+/K^+ homeostasis at the single-cell level in *Arabidopsis* and rice by quantifying sodium contents in root hairs and protoplasts (Halperin and Lynch 2003; Kader and Lindberg 2005). No reports have yet been made about the suitability of fluorescent dye for measuring Na^+ content at the organ level.

Here, we evaluated CoroNa Green fluorescent dye with *Arabidopsis* root tips. This dye has a molecular weight approximately one half that of SBFI and shows increases in fluorescence intensity without a shift in emission wavelength when it binds Na^+ (Meier et al. 2006). Experiments were performed to determine the optimum assay conditions for measuring Na^+ content *in planta*. We showed that CoroNa Green fluorescent dye is effective and convenient for measuring Na^+ content in roots of plants and demonstrated that CoroNa Green imaging is a useful technology for the study of ion homeostasis mechanisms in plants.

Materials and Methods

Tissue Preparation

Following cold treatment, seeds of *Arabidopsis thaliana* were sown on an MS medium and grown vertically for 5 days under long-day condition at 22°C. Five-day-old seedlings were treated with 0, 10, 50, 100, or 200 mM NaCl (pH 6.7) in MES buffer (0.6%) for 3 days in the same growth room where they had been previously placed. For the *salt overly sensitive1* (*sos1*) mutant and wild-type (*gl*) *Arabidopsis*, 7-day-old seedlings were treated with 0, 50, or 100 mM NaCl (pH 6.7) in MES (0.6%). Before dye-loading, NaCl-treated or untreated seedlings were washed three times with distilled water.

Dye-Loading

The CoroNa Green AM (Molecular Probe, Carlsbad, CA, USA) cell-permeable sodium indicator was used to visualize intracellular Na^+. Once the dye enters the cell, acetate moieties are cleaved by intracellular esterases, a modification that converts the probe into its sodium-responsive form. Upon binding sodium in the cell, the CoroNa Green becomes fluorescent. To determine the optimal concentration for use, 100 mM NaCl-treated seedlings were stained for 3 h at room temperature (RT) with 0, 5, 25, or 50 μM CoroNa Green that was diluted from a 1-mM stock solution in demethylsulphoxide (DMSO; Sigma). To measure sodium content *in planta*, we incubated NaCl-treated or untreated seedlings with 50 μM CoroNa Green for 3 h at RT under darkness. These dyed seedlings were then washed three times with distilled water to remove residual staining solution. Root tips were cut into 1-cm segments from intact seedlings and placed on glass slides with a small amount of water before being covered with a cover glass.

Detection of Na^+ Using CoroNa Green and Confocal Microscopy

CoroNa Green-dependent fluorescence was observed under a confocal laser scanning microscope (Fluoview FV300; Olympus, Tokyo, Japan). This dye has absorption and fluorescence emission maxima at approximately 492 and 516 nm, respectively. A 488-nm excitation laser source was used and emission wavelengths between 505 and 525 nm passed through an FITC (fluorescein isothiocyanate) green fluorescence filter; a DIC (differential interference contrast) bright transmission filter was employed to capture images of root tips. To prevent visualization artifacts for each sample set, their images were captured with equal photomultiplier tube (PMT) settings, in which the control sample (0 mM NaCl) showed no green fluorescence. Those images were used for quantifying Na^+ content within cells.

Quantification of Na^+ Intensity

Average fluorescence intensity was calculated after subtracting for the corresponding image background of each sample detected with an FITC filter. To obtain quantitative data, pixel values over a line (100 μm) manually located on the image were averaged using Fluoview FV300 imaging software (Olympus). These data were generated from the average number of pixels and means±SE (n=5). For *sos1* and wild-type *Arabidopsis*, fluorescence intensity was normalized to 0 mM NaCl (F/F_0) and plotted against concentrations of 50 and 100 mM NaCl.

Results and Discussion

Determination of Optimal Conditions for Measuring Na^+ Content *in planta* Using CoroNa Green

Fluorescence indicators are valuable for nondestructive monitoring of the spatial and temporal distribution of sodium. The newly developed CoroNa Green is already a useful tool for measuring intracellular Na^+ in animal cells and in starch granules isolated from reed (Meier et al. 2006;).

To determine the best conditions for CoroNa Green detection of Na^+ *in planta*, we pretreated *Arabidopsis*

seedlings with 100 mM NaCl, then incubated them with different concentrations of CoroNa Green to measure green fluorescence intensity in leaves, vascular bundles, and roots using FITC filter. The results showed that fluorescence was detected in plants incubated with a minimum of 25 μM CoroNa Green. Furthermore, there was little difference in fluorescence intensity in root tips loaded with various concentrations of dye, from 25 to 100 μM CoroNa Green (Fig. 1a). Therefore, we selected 50 μM as the concentration for further experiments. In plants pretreated with NaCl and incubated with 50 μM CoroNa Green, fluorescence was more uniformly and intensely distributed in the root tips than in other organs; none was observed in leaves. Interestingly, the vascular bundle showed a consistent intensity regardless of the concentration of NaCl pretreatment (Fig. 1b). This might suggest that endogenous sodium within the vascular bundle is maintained at constant levels regardless of different NaCl treatment.

It is generally difficult to load dyes into live cells for the detection of Na^+ *in planta* (Martin et al. 2005). Because SBFI and other fluorescent dyes, e.g., fura-2, that bind cations show low loading efficiency, researchers must use root protoplasts isolated from rice or mesophyll protoplasts from tobacco when measuring Na^+ contents under salt stress in those tissues (Halperin and Lynch 2003; Duan et al. 2007). Here, however, in this experiment the detection of Na^+ in *Arabidopsis* root could be conveniently obtained from intact root tissues loaded with 50 μM CoroNa Green. Therefore, this dye exhibits relatively high loading efficiency, at least in the root tips, making it suitable for monitoring Na^+ contents *in planta.*

Taken together, our data indicate that 50 μM CoroNa Green is effective for consistent detection of root-tip Na^+ *in planta*. Previous reviews reported that determination of Na^+ concentrations requires stable intracellular dye concentrations and permanent dye delivery. For example, intact epithelial sheets require higher dye concentrations and longer incubation times (Negulescu and Machen, 1990). In this experiment, we used *Arabidopsis* as a model plant to determine the optimal conditions of CoroNa Green treatment. This method will, however, necessitate validation and possible adjustments if applied to other plant species.

CoroNa Green Fluorescence Intensity Increases with Na^+ Concentration in *Arabidopsis* Root Tips

In order to test whether CoroNa Green is suitable for measuring the relative concentration of Na^+ *in planta*, *Arabidopsis* root tips were pretreated with 0, 10, 50, 100, or 200 mM NaCl before loading with 50 μM CoroNa Green. As shown in Fig. 2a, the fluorescence intensity in root tip gradually increased as the concentration of NaCl pretreatment increased, up to a concentration of 100 mM NaCl.

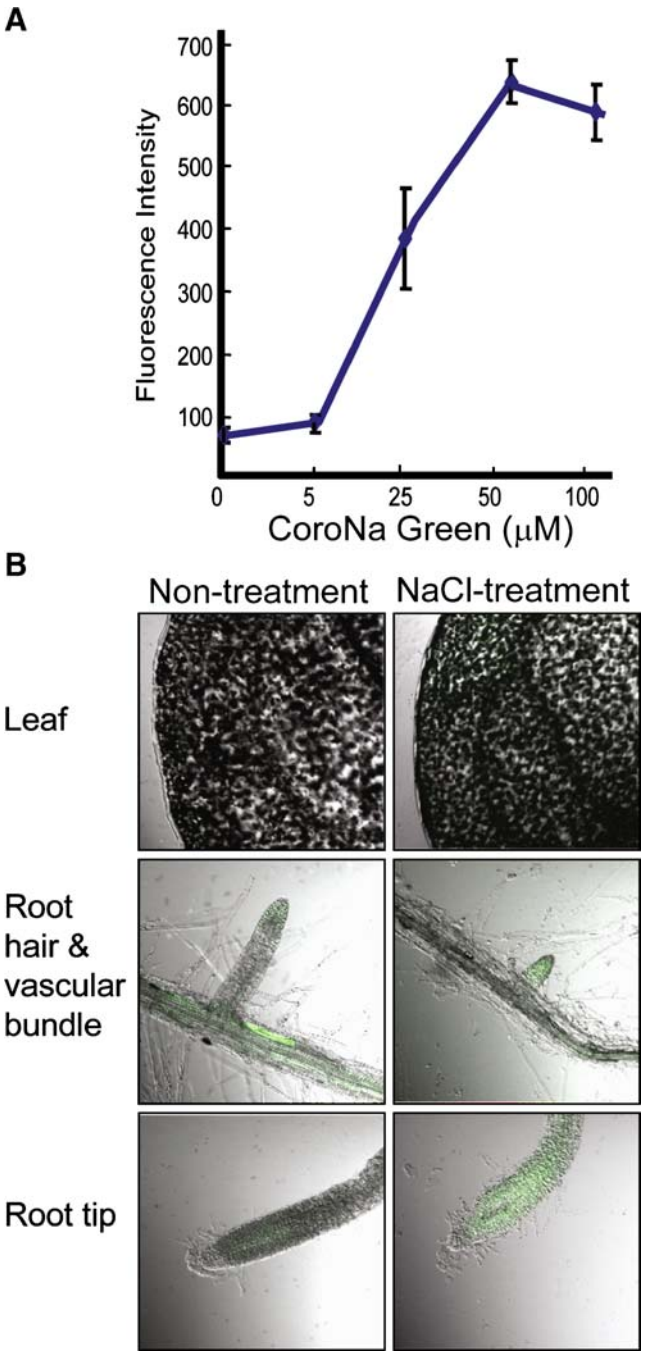

Fig. 1 Determination of optimum CoroNa Green concentration for detecting Na^+ in *A. thaliana*. **a** *Arabidopsis* roots pretreated with 100 mM NaCl were incubated with 0, 5, 25, 50, or 100 μM CoroNa Green for 3 h, and the fluorescence intensity was measured. Data represent mean intensity±SE for samples after subtracting for corresponding image background (n=4). The experiments were done with three sample sets, independently. One set included four samples; the data showed the means±SE (n=4). **b** NaCl-treated or nontreated *Arabidopsis* loaded with 50 μM CoroNa Green were observed to visualize fluorescence in each organ, leaf, vascular bundle, and root, using confocal microscopy with FITC and DIC filters

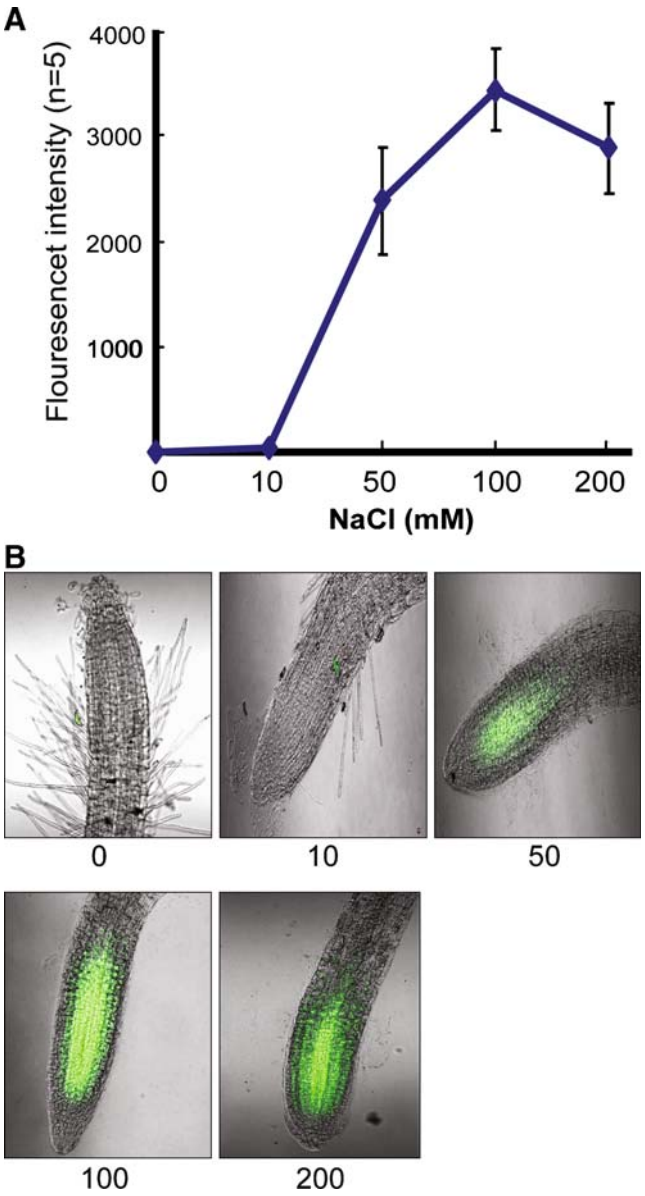

Fig. 2 CoroNa Green fluorescence intensity dose response to various concentrations of Na^+ in *Arabidopsis* root tips. **a** CoroNa Green-dependent fluorescence intensity in *Arabidopsis* root tips pretreated with NaCl concentrations of 0, 50, 100, 150, or 200 mM were observed using confocal microscopy. The fluorescence intensity was calculated as the average of each sample after subtraction of the corresponding image background. The experiments were done three times, independently. Each time, five samples were detected, and the graph showed the means±SE (n=5). **b** Changes in CoroNa Green fluorescence in root tips treated with 0, 10, 50, 100, or 200 mM NaCl (1, 2, 3, 4, or 5, respectively). Images were captured using a confocal laser scanning microscope (Olympus FV300) under equal PMT

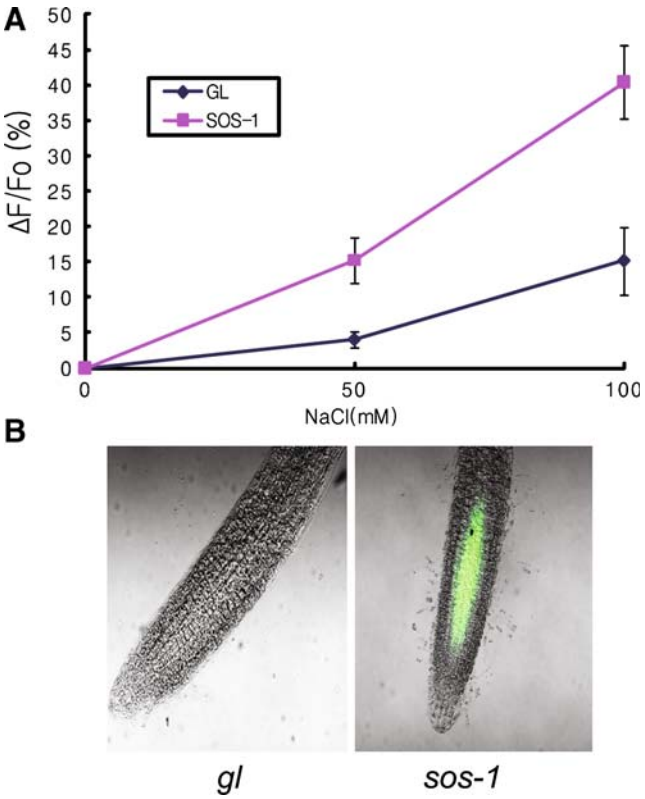

Fig. 3 Comparison of sodium contents between salt-sensitive mutant (*sos1*) and wild-type (*gl*) *Arabidopsis* root tips using CoroNa Green fluorescence. CoroNa Green fluorescence in root tips of *sos1* and wild-type (*gl*) *Arabidopsis* were detected using a FITC filter with identical PMT laser power (501 mV). **a** Changes in CoroNa Green fluorescence intensity normalized to 0 mM NaCl (F/F_0) were plotted against NaCl concentration (50 or 100 mM). The experiments were done two times with independent cultivations. The data show the average of each sample (n=6) from one of them. **b** The image of *sos1* and wild-type root tip pretreated with 50 mM NaCl were captured using a confocal laser scanning microscope (Olympus FV300) with FITC and DIC filters under with identical PMT (501mV)

The fluorescence increased several fold upon binding of Na^+ to CoroNa Green. However, at concentration 200 mM NaCl, fluorescence intensity was less than that observed in cells treated with 100 mM NaCl, indicating that this dye is suitable for conditions equal to or less than 150 mM NaCl treatment.

When *Arabidopsis* seedlings were treated with 100 mM NaCl for 2 days, the sodium content of the whole seedlings was almost 3.5-fold greater in dry weight (35 μg/mg dry weight) when compared to untreated plants (data not shown). The average concentration of Na^+ in *Arabidopsis* cells under normal conditions is less than 1%. Considering these ranges in Na^+ concentration, CoroNa Green is suitable as a Na^+ indicator for plants grown both under normal and salt-stress conditions thus limiting the usefulness of this dye for high-salt (200 mM NaCl) condition.

Confocal images were used to measure fluorescence intensity of Na^+ in cells. SBFI, a Na^+ indicator typically used for plant cells, requires a UV-adapted microscope, since the fluorescence intensity ratio comprises two different emission wavelengths (350 and 385 nm). In contrast, CoroNa Green fluorescence does not exhibit a shift in emission wavelengths when it binds Na^+, and its fluorescence increases upon Na-binding under blue light

(excitation wavelength). Thus, the fluorescence intensity from confocal images is sufficient to quantify cellular Na^+ contents (Fig. 2b). In animal cells, CoroNa Green also reliably represents cellular Na^+ changes and is suitable for measuring Na^+ alternations that are expected to occur during physiological processes (Meier et al. 2006). Here, we suggest that the fluorescence intensity detected with CoroNa Green is proportional to the Na^+ content in living plant cells. Thus, this dye is a useful Na^+ indicator for applications *in planta.*

Using CoroNa Green to Compare Sodium Uptake Between Salt-Sensitive Mutant (*sos1*) and Wild-Type (*gl*) *Arabidopsis*

To determine whether CoroNa Green is a good tool for comparing Na^+ contents among plants, we measured its green fluorescence from wild-type *gl Arabidopsis* plants and an *sos1* mutant that is hypersensitive to Na^+. We hypothesized that the latter would demonstrate more fluorescence intensity because it is reported to have a higher sodium content than wild type (*g1*) when grown under high-NaCl conditions (Wu et al. 1996).

Both *sos1* mutant and wild-type seedlings were treated with 0, 50, or 100 mM NaCl for 2 days, then incubated with 50 μM CoroNa Green. Upon addition of NaCl, the fluorescence intensity of both cultivars linearly increased (Fig. 3a). Confocal fluorescence microscopy revealed that *sos1* mutant exhibited much stronger fluorescence than that of wild type, when comparing plants treated with 50 mM NaCl and analyzed with identical PMT laser power (501 mV; Fig. 3b). In nontreated conditions (0 mM NaCl), wild-type seedling intracellular fluorescence was barely detected, while *sos1* seedlings showed bright fluorescence.

These results suggest that CoroNa Green would be useful as a tool for identifying sodium-sensitive mutants, using intact plants. Further, this dye can be applied to compare Na^+ content among plants. Similar experiments using SBFI were conducted with rice protoplast to compare sodium uptake between salt-sensitive and salt-tolerant cultivars (Kader and Lindberg 2005).

This study represents the first report of the use of CoroNa Green to measure intracellular Na^+ in intact plant cells. CoroNa Green is a suitable and convenient Na^+ indicator dye for use in experiments aimed to elucidate the sodium homeostasis mechanism in plants.

Acknowledgements This work was supported by Cooperative Research Projects for Bioenergy Crop Development (RIMS20070201036026), the Biogreen21 Project (RIMS200901FHT020813547) from RDA, and a grant (CG3134) from the Crop Functional Genomics section of the 21st Century Frontier Research Program.

References

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in *Arabidopsis*. Science 285:1256–1258

Cheong MS, Yun DJ (2007) Salt-stress signaling. J Plant Biol 50:148–155

Duan XG, Yang AF, Gao F, Zhang SL, Zhang JR (2007) Heterologous expression of vacuolar H^+-PPase enhances the electrochemical gradient across the vascular membrane and improves tobacco cell salt tolerance. Protoplasma 232:87–95

Halperin SJ, Lynch JP (2003) Effects of salinity on cytosolic Na^+ and K^+ in root hairs of *Arabidopsis thaliana*: *in vivo* measurements using the fluorescent dyes SBFI and PBFI. J Exp Bot 54:2035–2043

Kader MA, Lindberg S (2005) Uptake of sodium in protoplasts of salt-sensitive and salt-tolerant cultivars of rice, *Oryza sativa* L., determined by fluorescent dye SBFI. J Exp Bot 56:3149–3158

Martin V, Rothe A, Gee KR (2005) Fluorescent metal ion indicators based on benzoannelated crown systems: a green fluorescent indicator for intracellular sodium ions. Bioorg Med Chem Lett 15:1851–1855

Meier SD, Kovalchuk Y, Rose CR (2006) Properties of the new fluorescent Na^+ indicator CoroNa Green: comparison with SBFI and confocal Na^+ imaging. J Neurosci Meth 155:251–259

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250

Negulescu PA, Machen PE (1990) Intracellular ion activities and membrane transport in parietal cells measured with fluorescent dyes. In: Fleischer S, Fleischer B (eds) Methods in enzymology, vol 192. Academic, New York, pp 38–81

Park JY, Kim YY, Martinoia E, Lee Y (2008) Long-distance transporters of inorganic nutrients in plants. J Plant Biol 51:240–247

Shi H, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat Biotechnol 21:81–85

Wu SJ, Ding L, Zhu JK (1996) *SOS1*, a genetic locus essential for salt tolerance and potassium acquisition. Plant Cell 8:617–627

Zhang HX, Hodson JN, Williams JP, Blumwald E (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 U S A 98:12832–12836