Mini-Review

Ion flux profiles and plant ion homeostasis control under salt stress

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The ability of a plant to maintain an ionic homeostasis is crucial in plant salt tolerance. Direct evidence based on data from the non-invasive measurement of ion fluxes would not only offer new insight about the function of the transporter but also provide a whole plant approach for dissecting salt adaptation mechanisms. Here, we review some reports using the ion-selective microelectrodes to characterize the net ion fluxes of tissues or cells.

Introduction

The increasingly agricultural and environmental problems (including soil secondary salinization) caused by soil salinity on a worldwide scale have received much attention. Nearly 20% of the world’s cultivated land and nearly half of all irrigated lands are affected by salinity.1 One coping strategy is to select and breed salt-tolerant plants for areas where irrigation water is saline or soils are prone to salt buildup. To understand how plants tolerate and acclimate to saline environments is of great importance for genetic modification and plant selection. In general, salinity causes direct and indirect stresses on plant tissues, e.g., (1) a reduced water availability, due to the high soluble salts in the soil; (2) ion-specific effects, resulting from the excessive accumulation of toxic ions in plants (Na+ and Cl-); and, (3) the salt-induced secondary stress, e.g., oxidative bursts that caused by over-production of ROS (reactive oxygen species).1-4 Buildup of salt ions contributes to decreasing cellular osmotic potentials but this makes plants confront ion toxicity and oxidative stress. Therefore, whether plants could survive salinity is dependent to a large extent on the ability to retain the ionic homeostasis under saline conditions. Among the altered ion relations, the maintenance of low Na+ and Cl-, as well as keeping high concentrations of nutrition elements, especially K+ homeostasis, are crucial traits for plant salt adaptations.1-3

There are several approaches to explore ion relations at tissue and cellular levels, e.g., flame photometry, ion chromatography, X-ray microanalysis and ion selective microelectrodes (impalement).5-10 However, these traditional methods give us a concentration of elements that generally presents static information, dynamic flux of salt ions enable us to understand the detailed mechanisms of how plants control ion homeostasis in face of salinity. In recent years, the non-invasive measurement of ion fluxes is becoming an important approach to elucidate the dynamic changing of ion relations that caused by salinity.11,12 Up to the present, there are two typical but similar systems that designed to measure ion fluxes across membranes, i.e., the SIET (Scanning Ion-selective Electrode Technique) and the MIFE (Microelectrode Ion Flux Estimation) technique.11,12 These systems are proposed to measure electro-chemical potential differences between two positions close to the tissues or cells by moving the ion-selective microelectrodes in corresponding measuring solutions. Then the net fluxes of target elements are calculated according to the ion gradients which are converted from the measured electrochemical potentials (reviewed in refs. 11 and 12). The SIET and MIFE techniques supply a powerful tool to clarify the kinetic flux profiles which are necessary to illuminate the ion homeostasis regulation in salinised plants. In this review, we summarize the recent advances in ion flux investigations related to Na+, H+, K+ and Cl-, the major elements that affecting plant ionic status under salt stress.

Na+ Fluxes

Na+ uptake, transport and compartmentation are crucial for plants to survive saline environments with high NaCl content. In addition to the inhibition of Na+ influx, there are two major strategies to ameliorate Na+ toxicity in the cytosol: (1) improving vacuolar Na+ compartmentation via tonoplast Na+/H+ antiporters, and (2) increasing active Na+ extrusion to the external environment through Na+/H+ antiporters that located in the plasma membrane (PM).1-3 By means of the MIFE technique, Shabala,13 found a two-phase response for the net Na+ flux upon NaCl shock, i.e., an drastic influx of Na+ was registered immediately after NaCl
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was added, however, this net influx decreased dramatically and, with several minutes, plants usually exhibited a net Na+ efflux. Babourina et al. conclude that the Na+ efflux is performed by a Na+-extrusion system. However, Shabala, mentioned that measurements of Na+ were complicated by the low signal-to-noise (S/N) ratio for liquid ion exchanger (LIX) used at such high external ion concentrations. Methodological experiments conducted by Chen et al. showed that there were at least two issues complicating the transient Na+ flux analysis: (1) Na+ flux noise was much greater than for other cations, due to the high Na+ background level, and (2) Na+ selective microelectrodes were unsuitable for screening Na+ flux profiles because of non-ideal selectivity of the commercially available Na+ LIX, which was sensitive not only to Na+, but also to K+ and Ca2+.

Recently, we used the SIET to study the salt-induced alterations of root Na+ flux at both tissue and cellular levels in popular species. Na+ flux profiles were recorded in a measuring solution containing low concentration of Na+, and so the transient Na+ flux response to NaCl shock was not attempted. In that study, we compared ion flux profiles from two Populus species, contrasting in their salt tolerance, after prolonged exposure to salinity. This is necessary to clarify plant adaptations to long durations of salinity; so far, most of reports on salinity effects on root ion fluxes were obtained for acute stress conditions. Our results show that P. euphratica roots retained a higher capacity to extrude Na+ after a short term (ST) exposure to 50 mM NaCl (24 h) and a long term (LT) in a saline environment of 100 mM NaCl (15 d), as compared to P. popularis. The same trend was shown in root protoplasts, isolated from the LT-stressed P. euphratica roots. The NaCl-induced Na+/H+ exchange in root tissues and cells was inhibited by amiloride (a Na+/H+ antiporter inhibitor), indicating that the Na+ extrusion in stressed P. euphratica roots is the result of an active Na+/H+ antiport across the PM.

In our SIET studies, the flux measurements were carried out in a measuring solution with a lower Na+ (0.9 mM), in which a higher S/N ratio for the LIX was usually obtained. Our measurements of Na+ efflux in ST and LT stressed P. euphratica may not be interfered by the K+ and Ca2+ efflux. Using the K+ and Ca2+ selective microelectrodes, we measured fluxes of K+ and Ca2+ along the root axes at the same experimental conditions as used for Na+ flux measurements, but there was no marked K+ or Ca2+ efflux along the root axes in salinised P. euphratica (Sun J and Chen S-L, unpublished data). Therefore, we conclude that the K+ and Ca2+ efflux accompanying with the Na+ efflux induced by the NaCl stress has little influence on the actual Na+ fluxes in our experiments.

In our work, the root segments used for ion flux measurements were sampled from plants which were subjected to a period of saline treatment. In order to decrease the effect of salt unloading (Na+, Cl-) on flux recording, roots were rinsed with re-distilled water and immediately incubated in the measuring solution to equilibrate for 30 min, prior to the flux recording. The net Na+ efflux in equilibrated roots of P. euphratica was not resulted from a Na+ release from the cell wall (the release of Na+ from cell walls might be true for the transient responses, but not for steady-state fluxes which will saturate the Donnan system within 10–15 min after). To prove this point, we performed pharmacological experiments at tissue and cellular levels. Results showed that the salt-induced Na+ efflux was correspondingly inhibited by the application of amiloride, the inhibitor of Na+/H+ antiporter. Therefore, the experimental protocols adopted in our studies (i.e., sampling roots from salt-adapted plants, measuring root Na+ fluxes after a 30-min equilibration in measuring solution, increasing S/N ratio by reducing amount of Na+ in the measuring solution) is an alternative way to characterize the Na+ fluxes in stressed plants, due to the low S/N ratio of the Na+ LIX at high Na+ background level.

H+ Fluxes

H+ fluxes present a direct evidence of ion exchange coupling with H+, although there are several types of secondary transport systems located at the PM, e.g., Na+/H+ antiporters, H+/Cl- symporters, H+/K+ symporters. Furthermore, H+ selective microelectrode offers a more convenient and immediate manner approaching the Na+/H+ antiport that driven by H+-ATPase, as compared to the traditional method by measuring the dissipation of ΔpH (i.e., a Na+-induced increase in quinacrine fluorescence). It is suggested that NaCl stress induces a stimulation of H+-ATPases, which provides a driving force for PM Na+/H+ antiporters to move Na+ from the cytoplasm into the apoplast, thereby making an important contribution to the maintenance of low Na+ in the cytosol. Using the MIFE, a significant increase in net H+ efflux were recorded from the NaCl-shocked mesophyll tissues of a broad bean species (Vicia faba, salt-sensitive). The increased H+ efflux was inhibited by orthovanadate (a specific inhibitor of the PM H+ pump), indicating that PM H+-ATPase is involved in the cellular responses to salt stress.

It is worth to note that there are species-specific differences in H+ flux upon salt stress. A strong H+ efflux were observed in the root apex of sos1 mutants (Arabidopsis) given the NaCl shock; a general result of NaCl activation of the PM H+-ATPase pump. In contrast, NaCl induced a similar H+ flux response in sos2, sos3 and wild type, with an initial increase in the H+ influx into the root, implying an increased activity of Na+/ H+ antiport (extruding Na+ from the cell in exchange for H+ influx). In our SIET studies, salt-shock induced an increase of net H+ efflux in P. euphratica roots but not in the salt-sensitive P. popularis. The H+ efflux was completely arrested by the pretreatment with orthovanadate, suggesting that the salt-resistant P. euphratica rapidly upregulated the PM H+-ATPase activity after subjected to the NaCl stress (Sun J and Chen S-L, unpublished data). When provided with a prolonged salt stress, H+ influx significantly increased in root axes and the derived protoplasts of P. euphratica, but there were no corresponding changes in P. popularis. Noteworthy is that the enhanced H+ influx was re-found when LT-stressed P. euphratica cells were re-salinised with 50 mM NaCl. The salt-induced H+ influx in P. euphratica was inhibited by amiloride (a Na+/H+ antiporter inhibitor) and orthovanadate (a PM H+-ATPase inhibitor), suggesting the contribution of H+ influx to Na+ extrusion after plants exposed to prolonged NaCl treatment.
**K+ Fluxes**

There is no doubt that the K+ homeostasis play a crucial role in the salt adaptation of plant cells. Salt-resistant plants usually maintain a higher K+ nutrition at both tissue and cellular levels. By means of the MIFE, Shabala and colleagues have established the correlation between K+ fluxes and the capacity for salinity resistance in barley cultivars. They found a very strong correlation between net K+ fluxes (measured from the 3-day-old barley roots after 40 min of treatment in various concentrations of NaCl) and physiological responses (growth rate, biomass, net CO2 assimilation, chlorophyll fluorescence, etc.) after a month of NaCl treatment. The differences of NaCl-induced K+ efflux between tolerant and sensitive cultivars were remarkable, with much higher K+ efflux measured from sensitive cultivars. Similar results were also observed in wheat cultivars. Accordingly, they proposed a convenient non-destructive tool for screening the salt-resistant plants, i.e., by comparing the NaCl-induced K+ efflux. Using the SIET, we found that the NaCl-induced root K+ efflux was lower in salt-resistant poplar than in salt-sensitive poplar, irrespective of NaCl shock or long-term treatments (Sun J and Chen S-L, unpublished data).

More recent investigations suggest that the salt-induced K+ fluxes are mediated by PM H+-ATPase and calcium. The salt-resistant barley cultivars have a higher H+-ATPase activity under NaCl stress and retain a more negative membrane potential, thus inhibiting the NaCl-induced K+ efflux through depolarization-activated outward-rectifying K+ channels (DA-KORCs). In our studies, we found that the salt-induced K+ efflux in salt-resistant *P. euphratica* was significantly elevated by the pretreatment of orthovanadate (inhibitor of PM H+-ATPase) (Sun J and Chen S-L, unpublished data). The membrane potential indicated by the DiBAC4(3) fluorescence became more positive after the inhibitor treatment (Sun J and Chen S-L, unpublished data). The result verifies that the K+ efflux induced by salt shock is regulated by the membrane potential, which is to a large extent dependent on the PM H+-ATPase activity. Calcium supplement or exogenous application of compatible solutes and polyamines are able to ameliorate the detrimental effects of salinity by restricting the K+ efflux through DA-KORCs or the non-selective cation channels. Furthermore, compared with the net K+ efflux induced by the NaCl stress, isotonic mannitol stress usually induces a net K+ influx from both leaf and root tissues. These results indicate that different ionic mechanisms are involved in perception of "ionic" and "osmotic" components of the salt stress.

**Cl- Fluxes**

Cl- toxicity is more important than Na+ toxicity in some woody species. However, Cl- flux response to salt stress is less investigated, as compared to intensive studies focusing on Na+. In a study using the recombinant fluorescent probe, Lorenzen et al. detected a Cl- efflux in NaCl-stressed transgenic Arabidopsis. They speculated that the transport of Cl- might be thermodynamically coupled with Na+ under salinity condition. An influx of Cl-, immediately after addition of NaCl, was observed in bean mesophyll tissue. Unfortunately, measurements of Cl- ions were complicated by the low S/N ratio for LIX used at such high external ion concentrations. Using the SIET, we investigated the Cl- fluxes induced by NaCl and the hyperosmotic stress in two polar species contrasting in their salt tolerance. The flux measurements were carried out by using Cl- selective microelectrodes in a measuring solution containing low Cl- concentration (0.5 mM) (This is to reach a higher S/N ratio for the LIX). After a prolonged NaCl treatment, Cl- efflux significantly increased in salt-resistant *P. euphratica* roots but not in the salt-sensitive polar. However, an enhanced Cl- influx was recorded from the protoplasts isolated from LT-stressed *P. euphratica* root tissues. We conclude that the root Cl- efflux in salinised *P. euphratica* is presumably, at least in part, the result of ion release from the apoplast. This is consistent with our previous findings that salinized *P. euphratica* retained a high Cl- concentration in root cells by blocking efficiently the salt radical translocation through both the symplastic and apoplastic pathways.

We have noted that there are marked differences between the two poplar species in Cl- flux given the mannitol treatment. For *P. populans*, hyperosmotic-stressed roots (85 mM mannitol, 24 h) exhibited a drastic Cl- efflux, and the same trend was observed in short-term stressed plants (50 mM NaCl, 24 h). However, Cl- efflux in *P. euphratica* roots was not significantly enhanced at a hyperosmotic stress, which differs from the effects of NaCl stress, suggesting that NaCl-induced alternations of root Cl- fluxes are mainly the result of ion-specific effects.

**Conclusions**

In conclusion, the non-invasive ion flux techniques (SIET and MIFE) are a powerful tool to investigate plant ion relations. It is widely accepted that the transient and dynamic flux profiles are of great importance for exploring ion transport mechanisms in plant cells, although more careful studies need to be carried out in the future.

Based on the published literatures, the majority of ion flux studies have been mostly carried out on model plant species (Arabidopsis) and crop cultivars. To extend this work to economically important woody species, e.g., *Populus*, is helpful to screen the salt-resistant poplar species for afforestation in saline areas. Our understanding of the salt tolerance of woody species has increased considerably in recent years. We found that salt-sensitive poplar species are usually unable to restrict the large accumulations of Na+ and Cl- in root and shoot tissues, resulting in significant increases in ROS production despite marked increases in the activities of antioxidant enzymes in leaves and xylem sap. As compared to salt-sensitive poplars, *P. euphratica* plants effectively restricted the salt accumulation and did not exhibit an oxidative burst in response to the NaCl treatments. We hypothesized that salt-tolerant *P. euphratica* attenuates oxidative stress by NaCl exclusion, thus enabling ROS homeostasis to be maintained under saline conditions. Generally, the less accumulated salts could occur for two possibilities: inhibition of uptake or enhancement of active extrusion. By means of the SIET, we conclude that the greater capacity for restricting salt buildup in *P. euphratica* is a
result of Na\textsuperscript{+}/H\textsuperscript{+} antiport, although the mechanisms for Cl\textsuperscript{−} efflux in LT-stressed *P. euphratica* roots is still obscure and needs to be discovered.

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