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Effects of $NaHCO_3$ stress on Na^+ absorption in tobacco (*Nicotiana tabacum* Linn.)

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Abstract

Soil alkalization and salinization are widespread environmental problems. Alkaline salt-affected soil is typically characterized by an accumulation of sodium carbonate and bicarbonate. Little attention has been paid to the ion absorption characteristics of a plant under $NaHCO_3$ stress. This study aims to compare $NaHCO_3$ stress with NaCl stress to determine transient ion exchange and Na^+ toxicity on root surfaces. A non-invasive ion flux technique was applied and laser scanning confocal microscopy was conducted to investigate the effects of $NaHCO_3$ on Na^+ absorption in salt-sensitive tobacco (*Nicotiana tabacum* Linn.). $NaHCO_3$ shock treatment significantly increased the net H^+ efflux compared with the NaCl shock and control treatments. At the same time, vacuolar pH from 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) staining increased from 5.3 to 6.6. Exposure of roots to $NaHCO_3$ for 3 d resulted in both higher membrane permeability and higher Na^+ flux. Na^+ efflux was not inhibited by sodium orthovanadate, an inhibitor of the plasma membrane. These results indicate that $NaHCO_3$ addition induces high H^+ efflux and intracellular alkalization. Destruction of membrane permeability and the ion transport system led to uncontrolled Na^+ flux. The effect of $NaHCO_3$ reduces the resilience of plants for Na^+ osmotic stress, and increases the harm to the plant.

Keywords: $NaHCO_3$ stress,· Cell activity,· Intracellular pH,· Na^+ flux,· H^+-ATPase.
Abbreviations: BCECF-AM: 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, MES: 2-morpholinoethanesulphonic acid, PEPCase: Phosphoenolpyruvate carboxylase, NADP-ME: NADP-dependent malic enzyme, H^+-Ppase: H^+-pyrophosphatase, PM: Plasma membrane, FDA: Fluorescein diacetate, PI: Propidium iodide, SIET: Scanning ion-selective electrode technique.

Introduction

Salinity problems are compounded when the affected soils are also alkaline. An estimate of the cultivated area in the world showed that approximately 0.56×10^9 ha (37%) of the soil is sodic (Tanji, 1990). $NaHCO_3$ and Na_2CO_3 are the major alkaline mineral salts of sodic soils. A number of reports have demonstrated the existence of alkaline salt stress (Campbell and Nishio, 2000; Hartung et al., 2002; Rao et al., 2008; Zhang and Mu, 2009). The high pH surrounding roots directly causes some ions, such as Ca^{2+}, Mg^{2+}, and $H_2PO_4^-$, to precipitate (Yang et al., 2008b) and may endanger the function of plant roots. Salt stress usually disturbs ion homeostasis, resulting in oxidative bursts and salt damage (Wang et al., 2008). Reducing Na^+ influx, compartmentalizing Na^+ into the vacuole, and extruding Na^+ to the apoplast are three important strategies of plant cells to avoid excessive Na^+ accumulation in the cytosol (Sun et al., 2009b). Plants should retain lower cytosol Na^+ (Blumwald et al., 2000; Zhu, 2003). Plasma membrane (PM) Na^+/H^+ antiporters have been widely considered to play a crucial role in active Na^+ extrusion under saline conditions (Shi et al., 2000; Shi et al., 2002; Qiu et al., 2002; Martínez-Atienza et al., 2007). These PM Na^+/H^+ antiporters depend on electrochemical H^+ gradients generated by PM H^+-ATPase (Blumwald et al., 2000; Ma et al., 2002; Zhu, 2003). A large number of studies have focused on neutral salt stress in the selective absorption of the roots and Na^+ compartmentation on mixed saline stress (Yang et al., 2008b; Wang et al., 2011). There is still no system research on the role of selective absorption of Na^+ and H^+ ion pump under $NaHCO_3$ stress as we known. The response mechanism of halophytes and salt resistance crops on the morphological and physiological of alkaline salt stress has been extensive investigation. Such as halophytes of *Chloris virgata* (Yang et al., 2008a), *Suaeda glauca* (Bge.) (Yang et al., 2008b), *Aneurolepidium Chinese* (Trin.) Kitag (Shi and Wang, 2005), *Spartina alterniflora* (Poaceae) (Li et al., 2010), and salt resistance crops of sunflower、rice、wheat、 Proso millet and so on (Sheng et al., 2008; Shi and Sheng, 2005; Wang et al., 2011; Rao et al., 2008; Deng et al., 2011; Yang, 2012). The screening of these plants are based on salt resistance, which has an important role to reveal the alkaline salt stress and neutral salts stress and screen salt resistance plant. However, in order to reveal the transport mechanism and compartment of Na^+ under alkaline salt stress, salt-sensitive species would be a better choice. Tobacco (*Nicotiana tabacum* L.) is a salt-sensitive plant (Leatherwood et al., 2007), and it is also an important crop and model plant. Tobacco has been widely cultivation on Songnen Plain before it is salinization. However, to date, researches have very little published information available regarding tobacco under alkaline stress condition. In the present study, we use fluorescent probe and non-invasive ion flux techniques to measure root activity, intracellular pH, and fluxes of H^+ and Na^+ in the roots of $NaHCO_3$-stressed tobacco (*Nicotiana tabacum* Linn.) to compare and contrast NaCl-induced and

$NaHCO_3$-induced ion fluxes.

Results

$NaHCO_3$ induced a strong net H^+ efflux

As shown in Figure 1A, steady fluxes of H^+ were recorded for 5 min, and the average influx of H^+ was 0.66 pmol $cm^{-2}s^{-1}$. A salt shock was applied by adding a certain amount of salt stock (200 mM) to the solution. The final concentration of salt was 40 mM, and transient ion fluxes were monitored for another 30 min. This solution was added into a solution of $NaHCO_3$, resulting in 1 min to 2 min point fluctuations; increased H^+ efflux was also detected (Figure 1B). After 10 min to 15 min, the shock afforded by the H^+ efflux decreased and then stabilized gradually. Addition of $NaHCO_3$ and NaCl induced H^+ effluxes, with average values of 5.78 and 2.41 pmol cm^{-2} s^{-1}, respectively. $NaHCO_3$ induced a significantly higher H^+ efflux than NaCl after addition to the test solution (Figure 1C).

$NaHCO_3$ induced an intracellular increase in pH

A correlation curve of vacuolar pH in relation to the fluorescence ratio was constructed using the method of Krebs et al. (2010) with some modifications. Results in Figure 2A show a good linear relationship between pH and fluorescence ratio in the pH range from 5.4 to 7.4, and the equation of the recovery rate was found to be

$$y = 1.843x - 8.689 \quad (r^2 = 0.98).$$

In MS medium, the pH of root cell vacuoles remained between 5.1 to 5.4. When $NaHCO_3$ was added to the mixture, A pH range of root cell vacuoles was immediately monitored. The fluorescence ratio of root cells increased with time (Figure 2C), reflecting the increase in vacuolar pH from 5.3 to 6.6. The pH of the vacuoles increased rapidly in the 1-5 min after $NaHCO_3$ addition, followed by a more gradual increase. After 25 min, the variation in pH stabilized (Figure 2B). These results show that $NaHCO_3$ could induce a significant increase in the pH of cell vacuoles.

$NaHCO_3$ induced a strong net Na^+ efflux in 3 d treatment seedling

Ion flux measurements were started from the tip and went along the root axis until 1500 μm. In contrast to control, salt stress roots exhibited a marked Na^+ efflux along the root axis. The measuring site was 650 μm from the root tip, in which a vigorous difference of Na^+ flux was observed (Figure 3A). For the roots from salt stress, the length of the root tip was not similar to those of the controls but significant difference at the initial part of elongation zone. Afterward, salt stress of different concentration was observed in the elongation region of 500-700 μm depending on morphological difference. Under the same concentration of Na^+, the morphologies of tobacco seedlings in different salt treatment after 3 days were shown in Figure 3B. Significant reduction was observed on root length of tobacco seedlings under 48 mM NaCl +2 mM $NaHCO_3$ treatment. The inhibition of root growth was increased with the rising of $NaHCO_3$ concentration, and the seedling roots have stopped growing when the concentration of $NaHCO_3$ was more than 4 mM. Changes of net Na^+ flux with time in the root elongation zone were shown in Figure 3C. The lowest net Na^+ flux was observed on control solution with an average value of -10.86 pmol $cm^{-2}s^{-1}$, which reflected a normal Na^+ influx of root cell under the test solution of 0.9 mM Na^+ concentration. Net Na^+ efflux of root in 50 mM NaCl treatment was 201.97 pmol $cm^{-2}s^{-1}$, and the flow rate increased with the rising of the concentration of $NaHCO_3$. The net Na^+ efflux of root was up to 705.36 pmol $cm^{-2}s^{-1}$ under 40 mM NaCl + 10 mM $NaHCO_3$ treatment. With $NaHCO_3$ treatment, Na^+ efflux was significantly greater than the same concentration of NaCl treatment. However there was no significant difference of the net Na^+ efflux between adjacent concentrations of $NaHCO_3$ treatment. Ignored the control treatment, there was a good logarithmic relationship between pH value of external medium and the Na^+ efflux, the equation of recovery rate was

$$y = 0.854\ln(x) + 1.935 \quad (r^2 = 0.979).$$

Correlation analysis showed that efflux of Na^+ was impacted by the external solution pH greatly. Figure 3D showed the net Na^+ flux of seedling root treated by 48 mM NaCl +2 mM $NaHCO_3$ for 3 days, with or without sodium orthovanadate treatment before measure, and there was no significant difference between them. As sodium orthovanadate is a specific inhibitor of plasma membrane H^+-ATP enzyme, the results indicate that Na^+ efflux was not affected by plasma membrane H^+-ATP enzyme. As known from the point of classical thermodynamics, the Na^+ efflux was completely from the difference between Na^+ concentration of solution and root cell. The results show that the Na^+ concentration in root cells will increasing with the concentration rising of $NaHCO_3$, and $NaHCO_3$ will reduce the regulatory effect on selective absorption of Na^+.

Effect of $NaHCO_3$ on root cell activity

Roots from seedlings treated for 3 d were stained for 10 min with FDA or PI solution and visualized using a laser scanning confocal microscope. The roots of tobacco were readily and entirely stained by FDA (green fluorescence in Figure 4). A large number of root caps and epidermal cells were stained by PI in 6 mM $NaHCO_3$ solution (red fluorescence in Figure 4). With increasing concentrations of $NaHCO_3$, the number and scope of PI-stained cells increased. FDA passes through cell membranes and is hydrolyzed by intracellular esterases to produce fluorescence. These results indicate that root cells are able to maintain relatively high esterase activities in sodic conditions, considering that PI cannot pass through whole cell membranes. PM permeability was enhanced with increasing concentrations of $NaHCO_3$, indicating that membrane permeability was changed prior to the loss of hydrolase activity.

Discussions

NaCl shock increased the H^+ efflux, indicating activated PM H^+-ATPase and resulting in a corresponding acidification. Salt shock creates an acidic environment that is favorable for Na^+/H^+ exchange across the PM (Sun et al., 2009b). $NaHCO_3$ shock markedly increased the H^+ efflux, but the electrochemical H^+ gradient was difficult to maintain due to the high external pH. The change in pH from 5.3 to 6.6 indicates that the intracellular pH significantly increased; this increase was accompanied by the H^+ efflux. Cell alkalization caused by $NaHCO_3$ may bring about problems more severe than those caused by salt accumulation. Plants could regulate their internal pH to some extent by modifying the balance between PEPCase, NADP-ME, and H^+-Ppase activities (Zhu et al., 2011) to gradually adapt to high pH environments. This phenomenon could be proven by determining the activity of root cells. Roots immersed in different solutions of $NaHCO_3$

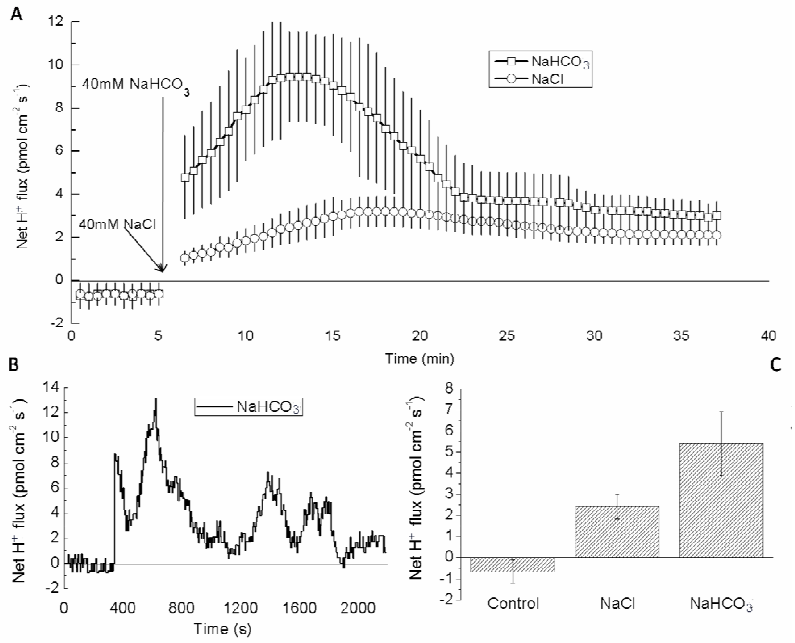

Fig 1. Effects of salt on transient H^+ kinetics at elongation region of tobacco. Before the salt, steady H^+ fluxes were monitored for 5 min. Then, H^+ kinetics was recorded after adding the salt into the measuring chamber. Each point is the mean of four individual plants and bars represent the standard error. (A) The average flux curves (B) Typical flux curve. (C) The average flux values.

for 3 d were subjected to FDA or PI staining. The roots of tobacco were readily and entirely stained by FDA (Figure 4), indicating that root cells could maintain high enzymatic activities and live states. PI staining resulted in red fluorescence appearing in roots treated with 6 mM $NaHCO_3$ solution. The numbers and scope of PI-stained cells increased with increasing concentrations of $NaHCO_3$. The contradiction between FDA staining and PI staining has been reported by Davey and Hexley (2011). Red fluorescence after PI staining is not necessarily representative of death but it appears to indicate membrane permeability caused by a stressful environment. The leaf electrolyte leakage rate observed during alkaline stress also suggests direct disruption of the structure of membrane selectivity (Shi and Yin, 1993; Shi and Sheng, 2005; Shi and Wang, 2005; Li et al., 2010). Further determination of the Na^+ flux also showed a significant change in membrane permeability under $NaHCO_3$ stress. The inhibitory effects of PM transport inhibitors on ion fluxes were examined. Roots were subjected to 500 μM sodium orthovanadate for 30 min (Sun et al., 2009a), but the increased Na^+ efflux could not be inhibited by sodium orthovanadate. This finding indicates that Na^+ efflux is a passive diffusion process. Na^+ extrusion is essential to sustain Na^+ homeostasis in the cytosol (Blumwald et al., 2000; Demidchik and Tester, 2002; Zhu, 2003). PM Na^+/H^+ antiporters have widely been considered to play a crucial role in Na^+ extrusion under saline conditions (Qiu et al., 2002; Ma et al., 2002; Martínez-Atienza, 2007). These PM Na^+/H^+ antiporters depend on the electrochemical H^+ gradients generated by PM H^+-ATPase (Blumwald et al., 2000; Zhu, 2003). The results suggest that alkaline stress increases Na^+ accumulation in the root, which agrees with the result of some authors (2011), who found that alkaline stress results in enhanced Na^+ accumulation. Both the change in membrane permeability and uncontrolled Na^+ transport are futile transports of Na^+ and thus do not contribute to growth (Kronzucker and Britto, 2011). The disappearance of the role of PM H^+-ATPase may also lead to the loss of potassium. The decline of this element in roots could act as a signal for the execution of a cell death program (Huh et al., 2002; Demidchik et al., 2010).

Materials and methods

Plant material and growth conditions

Tobacco seeds (*Nicotiana tabacum* L. Yun 87) are from the Yunnan Academy of Tobacco Science (Yunnan, China). Seeds of tobacco were sown in sterile petri dishes containing half-concentrated MS medium in 1% agar (adjusted to pH 6.5 with 1 M KOH). The petri dishes were kept vertically in a growth chamber at 22 ℃ under a 16h d^{-1} photoperiod and 180 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density of light intensity. For uniform-nutrient treatments, three-day-old seedlings were transferred to half-concentrated MS liquid medium (adjusted to pH 6.5 with 1 M KOH) for continuing to grow for 1 day. After 1 day, the seedlings were transferred to half-concentrated MS liquid medium containing the appropriate treatments. After 3 day treatment, analyses were conducted for root activity, Na^+ flux, and salt shock assays.

Measurement of root activity by FDA/PI staining

Root activity was measured in accordance with the method of Koyama et al. (2001). The root system from the seedlings was washed thrice with distilled water and placed into a glass dish with a fluorescein diacetate (FDA)/propidium iodide (PI) solution for 10 min (Jones and Senft, 1985; Truernit and Haseloff, 2008). PI was used as 10 μg ml^{-1} solution in water and FDA was used as a 5 μg ml^{-1} solution in water. Images of the root were taken within 30 min after staining. Excitation and collection wavelengths were examined using a Nikon C1 Laser scanning confocal microscope (Japan).

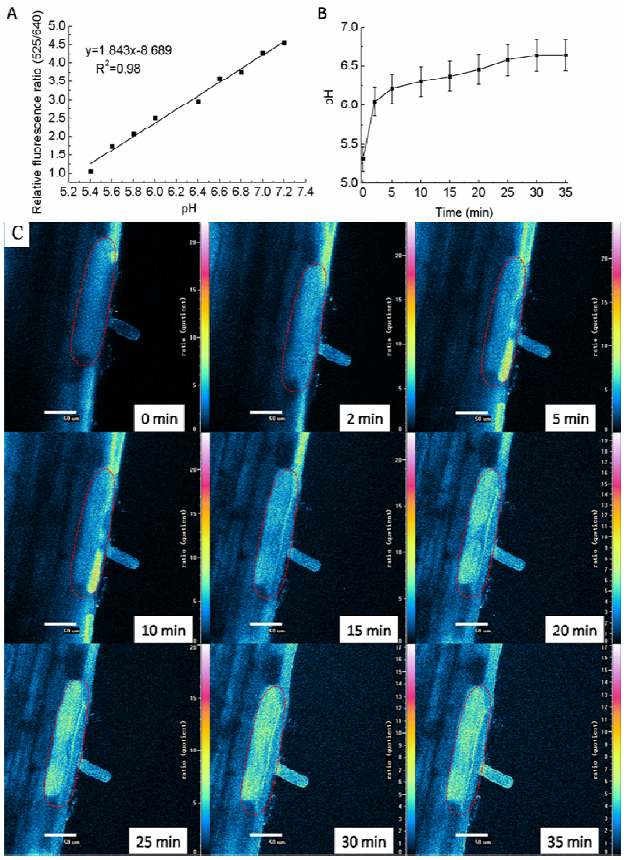

Fig 2. Photographs of simultaneous changes in fluorescence ratio (525/640) were recorded with the fluorescent probe BCECF. Changes of pH are expressed as the fluorescence ratio value, following excitation at 488nm, the intensity of BCECF fluorescence is linearly dependent on pH in the range of 5.0 to 7.6 (Musgrove et al., 1986, Rotin et al., 1987). 0min: no $NaHCO_3$, 2-35 min: Changes of fluorescence ratio between 2-35 min after adding $NaHCO_3$. (A) Calibration curve of pH (B) Change curve of vacuolar pH value after adding $NaHCO_3$ (C) Fluorescence ratio pictures within 35min.

Measurement of net Na^+ flux with the SIET system

Net fluxes of Na^+ and H^+ were measured non-invasively using the Scanning Ion-selective Electrode Technique (the SIET system, BIO-001A, Younger USA Science and Technology Corp., Amherst, MA; Applicable Electronics Inc., Forestdale, MA and Science Wares Inc., East Falmouth, MA). The principle of this method, the instrument and preparation of microelectrodes were detailed in Newman (2001) and Sun et al. (2009a). Seedlings were balanced in accordance with the method of Chen et al. (2005), and fixed in measuring chamber. Determination process and the calibration solution preparation were performed according to the method proposed by Sun et al (2009a), with slight modifications. The microelectrode was vibrated in the measuring solution between two positions, 5 µm and 35 µm from the root surface, along an axis perpendicular to the root. The net fluxes of Na^+ at the tip of root, meristem, elongation zone and mature zone were measured individually. When the measuring site was elongation zone of 500-700 µm from the root tip, a vigorous flux different of Na^+ was usually observed (from control and treatments). Subsequently, the middle of elongation zone (About 650 µm or so depending on morphological difference) was selected for determination of sodium flux from different treatment. Each plant was measured once. The final flux values at elongation zone were the means of 4-5 individual plants depending on the consistency of data from each treatment. Ion selective electrodes of the following target ions were calibrated prior to flux measurements:

(1) Na^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 7.0 adjusted with Tris and HCl

(2) Na^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 4.6mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 6.0 adjusted with Tris and HCl

The measuring solution was composed of 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5mM NaCl, 0.3 mM MES and 0.2mM Na_2SO_4, (pH 6.5 adjusted with Tris)

Transient kinetics upon salt shock

Transient H^+ kinetics response to salt was examined in elongation zone, which were about 650 µm from the root tip. Control roots were equilibrated in measuring solution for 20min. Thereafter, steady fluxes of H^+ were recorded for 5-6 min before the salt addition. Then, a salt shock was given by adding an acquired amount of salt stock (200 mM), a final concentration of salt was 40 mM and transient ion fluxes were monitored for a further 30 min. The data measured during the first 2-3 min were discarded due to the diffusion effects of stock addition (Shabala, 2000). Ion selective electrodes of the following target ions were calibrated prior to flux measurements:

(1) H^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 7.5 adjusted with Tris and HCl

(2) H^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 6.0 adjusted with Tris and HCl

Simultaneous measurement of intracellular pH

The measurements of vacuolar pH were performed by incubating roots with pH-sensitive dye (2',7'–bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, BCECF-AM) following protocols provided by the manufacturer (Invitrogen, Carlsbad, CA). The BCECF-AM was added to the culture medium to a final concentration of 5 µM. Roots from 3 d treatment were incubated for 15 min. Fluorescence was observed with a Nikon C1 Laser scanning confocal microscope running Nikon EZ-C1 2.3 software. Detection of BCECF-AM fluorescence used a fixed excitation at 488 nm and emission was measured from a ratio of the fluorescence emission at 525 nm (pH-dependent) to 640 nm (pH-independent) (Boyer and Hedley, 1994). A salt shock with 5 µM BCECF-AM was given by adding an acquired amount of $NaHCO_3$ stock (200 mM) and a final concentration of $NaHCO_3$ was 40 mM. Images of ratios were collected from time to time and monitored for a further 35 min. Control plants were continued to observe for 35 min without the addition of $NaHCO_3$. The ratio was then used to calculate the pH on the basis of a calibration curve. Calibration curve was obtained by in situ calibration according to method of Yoshida (1994) and Swanson and Jones (1996).

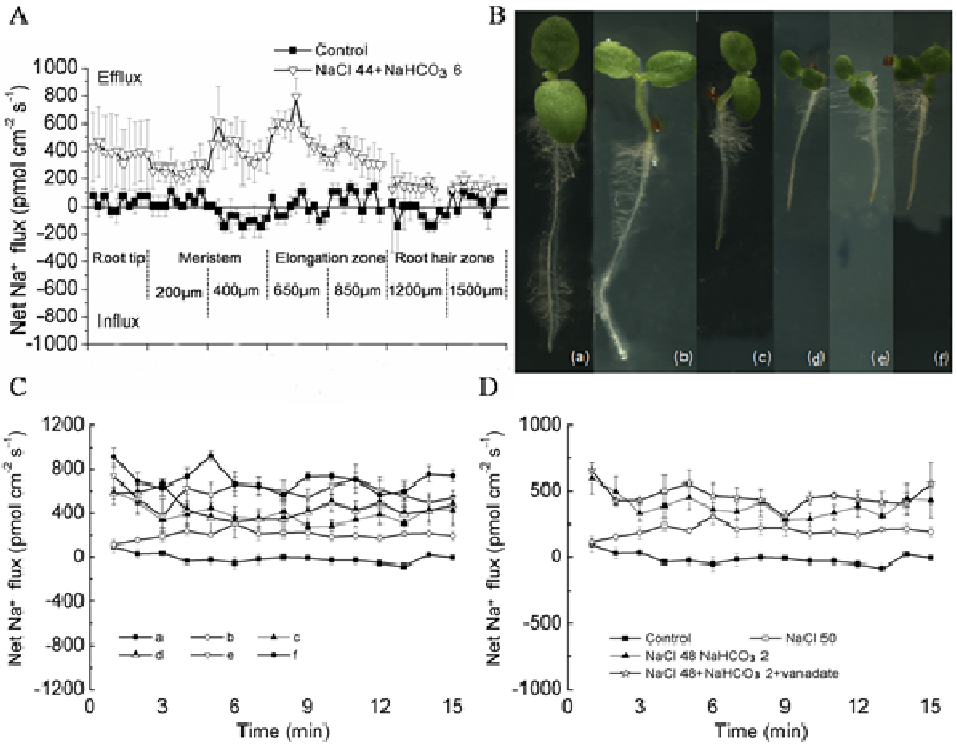

Fig 3. Effect of $NaHCO_3$ on net Na^+ flux of tobacoo (Nicotiana tabacum Linn.) from different salt treatment of 3d. (A) Effects of $NaHCO_3$ on Na^+ fluxes at root tip, meristematic zone (200μm, 400μm), elongation zone (650μm, 850μm) and root hair zone (1200μm, 1500μm). (B) Effects of $NaHCO_3$ on the morphologies of tobacco seedlings. (C) Effects of $NaHCO_3$ on Na^+ fluxes at elongation zon. (D) Effects of sodium orthovanadate on Na^+ fluxes at the root treated by NaHCO3. Each point reprents the mean of 4 individual roots and bars represent the standard error of the mean. (a) Control; (b) NaCl 50 mM; (c) NaCl 48 mM+$NaHCO_3$ 2 mM; (d) NaCl 46 mM+$NaHCO_3$ 4 mM; (e) NaCl 44mM+$NaHCO_3$ 6 mM; (f) NaCl 40 mM+$NaHCO_3$ 10 mM.

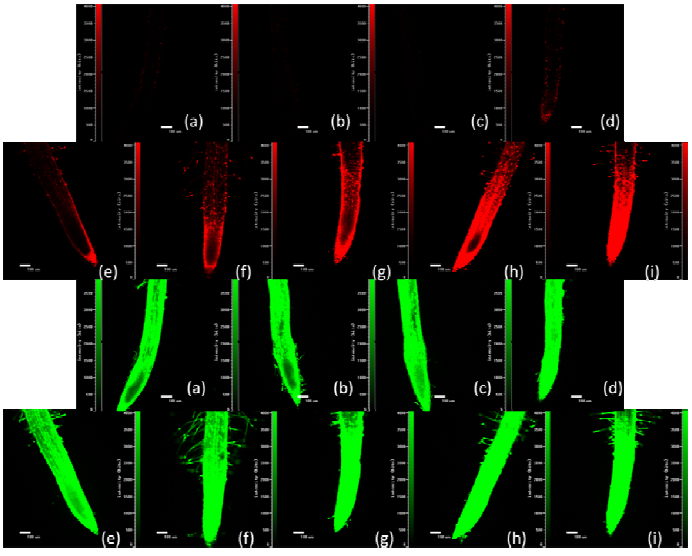

Fig 4. Effect of $NaHCO_3$ on root cell activity and membrane permeability. The pictures show a treatment for 3d on 1/2 MS medium containing the following concentration of $NaHCO_3$: (a) Control; (b) $NaHCO_3$ 1mM; (c) $NaHCO_3$ 3 mM; (d) $NaHCO_3$ 6 mM; (e) NaCl $NaHCO_3$ 10 mM; (f) $NaHCO_3$ 20 mM; (g) $NaHCO_3$ 30 mM; (h) $NaHCO_3$ 40 mM; (i) $NaHCO_3$ 50 mM. The red fluorescence from excited PI fluorescence and green fluorescence from excited FDA. The red dots denoted that nucleic acid had been stained by PI.

Statistical analysis

Data were analyzed using the statistical software SPSS 14.0 (SPSS Inc., Chicago, USA). H^+ flux, Na^+ flux, pH and root staining was represented by means and standard errors (S.E.) of 4-5 individual plants depending on the consistency of data from each treatment. Data of H^+ flux and Na^+ flux were examined using one-way ANOVA for each treatment. Multiple comparisons of means of data treatments within the plants were performed using Duncan's test at the 0.05 significance level.

Conclusions

Tobacco roots treated with $NaHCO_3$ displayed a higher H^+ efflux with intracellular alkalization, as evidenced by the salt shock experiment. EIET revealed that higher Na^+ exclusion is a non-dependent activity of PM H^+-ATPase. The root activity test showed that lower concentrations of $NaHCO_3$ (6 mM) induce changes in membrane permeability. These results indicate that high pH and salt content around roots after $NaHCO_3$ addition induce H^+ efflux and cell alkalization. These conditions are a serious problem that could destroy membrane permeability and ion transport system. The balance between Na^+ and K^+ was further disturbed in the presence of uncontrolled Na^+ flux.

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