Analytical Biochemistry 418 (2011) 295–297

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Modified noninvasive microtest electrophysiological technology for vacuolar H^+ flux detection

Xianyang Chen, Lingling Nie, Hexigeduleng Bao, Ping Jiang, Sulian Lv, Yinxin Li *

Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, People's Republic of China

ARTICLE INFO

Article history:
Received 20 September 2010
Received in revised form 16 July 2011
Accepted 16 July 2011
Available online 22 July 2011

Keywords:
H^+ flux
NMT
SeNHX1
Vacuole isolation

ABSTRACT

This paper describes a modified noninvasive microtest electrophysiological technology (NMT) for vacuolar H^+ flux detection. In this NMT system, the vacuole isolation procedure and buffer slope were modified, and the measuring errors from small spherical geometry were corrected. The trends in changes of vacuolar H^+ flux (ΔH^+ flux) after ATP or PP_i supply calculated by NMT were consistent with the activities of V-ATPase and PPase measured by traditional methods. These findings indicate that our modified NMT is an appropriate method for vacuolar H^+ flux detection.

Traditionally, H^+ transportation across tonoplast vesicles has been detected by pH fluorescence quenching *in vitro* [1]. However, although more complicated and fussy for isolation of high purity tonoplasts, it is not sensitive enough to accomplish a real-time detection of weak H^+ fluxes across membranes. Recently, a noninvasive microtest electrophysiological technology (NMT)[1] has been widely used in studies of plant electrophysiological processes [2]. The distinctive advantages of NMT for detecting vacuolar H^+ current include: (a) noninvasion to reduce fluctuation caused by mechanical damage; (b) high sensitivity to weak electric currents such as H^+ flux; and (c) real-time dynamic detection for the whole process [3]. Although Ca^{2+} fluxes in vacuoles of red beet (*Beta vulgaris* L.) have been calculated by NMT [4], little effort was made in using NMT to investigate the dynamic process of vacuolar H^+ flux. In this study, NMT was modified to detect vacuolar H^+ fluxes and to record its dynamic change processes under NaCl shock conditions.

First, vacuoles must be isolated from plant tissue, and we simplified the isolation procedure according to the characteristics of NMT. Since the operational area, including samples and electrode, is visible in NMT, we can distinguish and choose the appropriate vacuoles in the isolation solution. Therefore, it is not indispensable to obtain high purity vacuole collections using the traditional ultracentrifuge method [1]. A simplified procedure was then devised from the traditional method with the following modifications: (i) The suspension solution of released vacuoles was loaded on a layer consisting of 8% Ficoll-400, 25 mM hydroxymethylaminomethane–HCl (Tris–HCl) with pH 7.5 and 0.5 M mannitol, instead of on the top of a four-step discontinuous gradient. (ii) The suspension solution was centrifuged at 1000*g* for 30 min at 4 °C using a swinging bucket rotor instead of an ultracentrifuge at 90,000*g* for 2 h. (iii) Intact mature vacuoles were collected from the top layer instead of the interface between the 1.5% and the 7% (w/w) Ficoll-400 layer using an ultracentrifuge. The target vacuoles were immersed in the test solution and loaded on the test chamber for further H^+ flux measurement.

Then, we modified and normalized the distance between the electrode probe and the vacuole based on the NMT system (see Supplementary Information 1). The NMT system lacks a real-time scale, and the distance between the electrode probe and the small spherical sample is hard to maintain in different tests, leading to amplified measuring errors [5,6]. Therefore, in this study, the distance between the probe and the vacuole was recorded and calculated with analysis software Axiovision Rel 4.1. The H^+ flux data were not used for further analysis until the distances were less than 2 μm. When the diameters of vacuoles were more than 20 μm, and the moving range of the electrode probe was 10 μm with its size ranging from 2 to 4 μm, the measuring error from small spherical geometry was therefore very limited [5,6].

Furthermore, we corrected the values of H^+ flux calculated using the NMT system. In the NMT system, the H^+ flux was calculated automatically following Fick's law of diffusion. However, different from other ions, since H^+ always moves with buffer molecules, the H^+ flux value should be corrected in the NMT measurement [7]. According to the formula described by Porterfield et al. [7] (see

* Corresponding author. Fax: +86 10 82596139.
E-mail address: yxli@ibcas.ac.cn (Y. Li).

[1] *Abbreviations used:* ΔH^+ flux, the change of vacuolar H^+ flux; MES, 2-*N*-morpholinoethanesulfonic acid; NMT, noninvasive microtest electrophysiological technology; Tris, hydroxymethyl aminomethane; WT, wild type.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.07.018

Supplementary Information 2), we obtained the correction coefficient: $1 + B_t \times (\mathrm{LBC}/K_a)$. Here, B_t is the total buffer concentration in the bulk media, LBC is the linear buffering coefficient, and K_a is the disassociation constant of the buffer [7]. The test buffer used in the present study was 2-*N*-morpholinoethanesulfonic acid (MES) with pH 6.7; hence, B_t of H^+ is $10^{-6.7}$, the LBC is 0.25008, and K_a is 7.94×10^{-7} according to Porterfield et al. [7]. Therefore, the "correction coefficient" here was calculated as 1.062843. Finally, we corrected the values calculated using the NMT system by multiplying 1.062843.

To confirm the appropriateness of NMT for vacuolar H^+ flux detection, the transgenic tobacco line (TS15) overexpressing *SeNHX1* (vacuolar-type Na^+/H^+ antiporter gene from halophyte *Salicornia europaea*) was introduced [8]. Vacuoles were isolated from leaves of TS15 and the wild-type (WT) tobacco plants. The vacuolar H^+ fluxes under normal (0 mM NaCl) and 200 mM NaCl shock conditions were detected using NMT, where ΔH^+ flux (the change of H^+ flux after ATP or PP_i supply) was used to indicate the activities of V-ATPase and PPase, the energy metabolism enzymes.

Under normal conditions after ATP or PP_i treatment, as shown in Fig. 1, the dynamic curves displayed continuously increased net H^+ influx in vacuoles of TS15 and WT plants (Fig. 1A and B). The mean net H^+ flux also exhibited distinct influx in vacuoles of WT and TS15 (Fig. 1a and b).

Under 200 mM NaCl stress, the dynamic curves showed that the net H^+ effluxes into cytoplast increased in both transgenic and WT plants (Fig. 1C and D). However, TS15 exhibited an enhanced mean net H^+ efflux than WT (Fig. 1c and d), indicating higher NHX1 activities in transgenic plants. This result was consistent with our previous study that the leaf cell sap osmolality increased after NaCl stress, due to more Na^+ congregating in vacuoles of *SeNHX1* transgenic tobacco than in those of control [8].

Under 200 mM NaCl treatment, after the ATP or PP_i supply, the dynamic curves showed that the net H^+ influxes into vacuoles increased sharply in both transgenic and WT plants (Fig. 1C and D). The mean net H^+ flux was comparable between transgenic and WT plants after ATP supply, whereas the transgenic maintained a weak efflux compared with the distinct influx in WT plants after PP_i treatment (Fig. 1c and d).

Furthermore, the activities of energy metabolism enzymes represented by V-ATPase and PPase were indicated by changes of vacuolar H^+ flux (ΔH^+ flux) after ATP or PP_i supply in WT and TS15

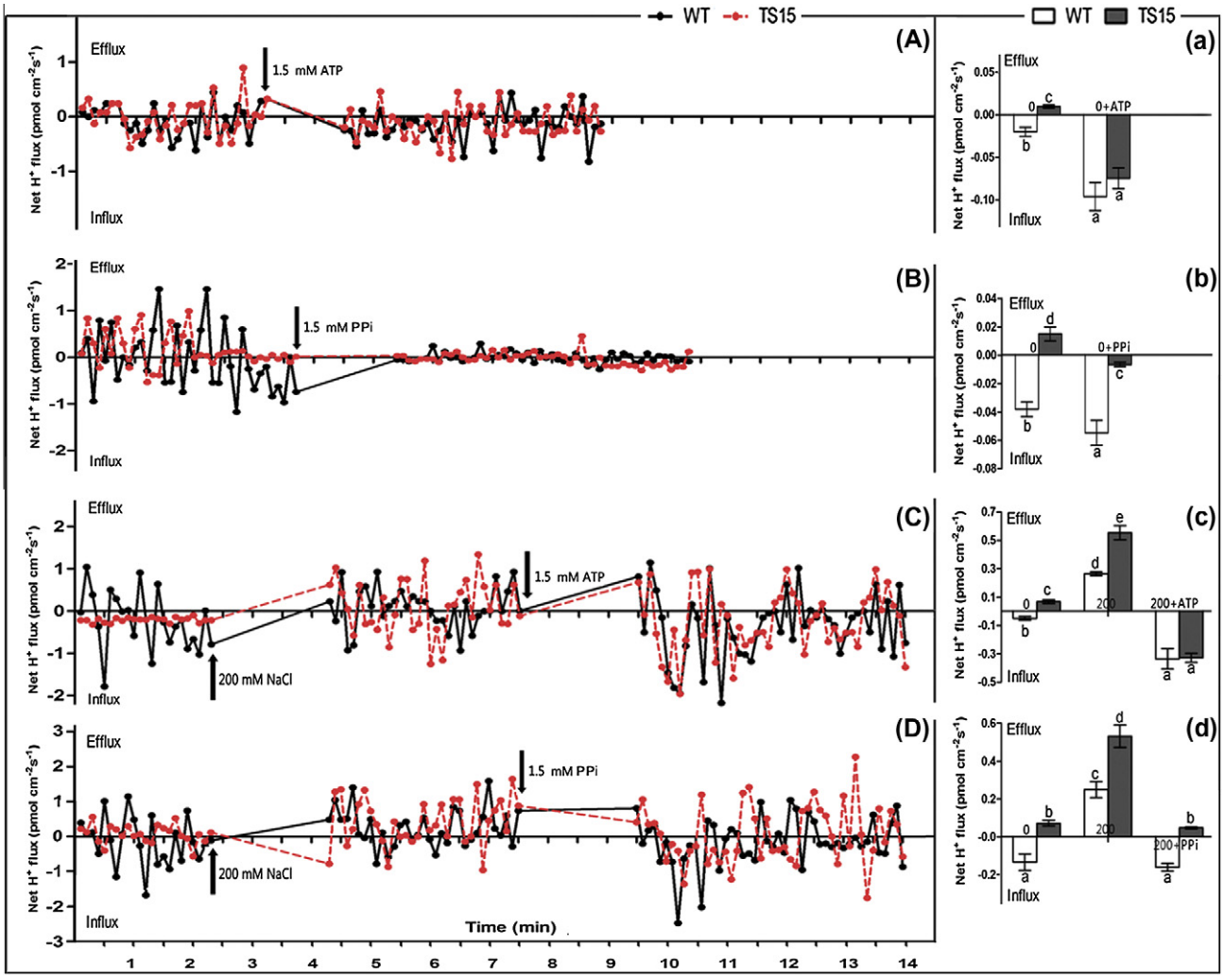

Fig.1. Vacuolar net H^+ fluxes in *SeNHX1* transgenic and WT tobacco under normal or 200 mM NaCl shock conditions. (A–D) Dynamic curves of transient net H^+ fluxes: normal condition plus supply of ATP (A) or PP_i (B), 200 mM NaCl treatment plus supply of ATP (C) or PP_i (D). (a–d) Mean of net vacuolar H^+ fluxes corresponding to (A–D) during the measuring periods. The value obtained from NMT indicates net ion flux; the positive values of H^+ flux in the figures represent cation efflux or anion influx and vice versa. Measurement of vacuole H^+ flux was started 1–3 min earlier before treatments and repeated in six vacuoles. WT, wild-type tobacco plant, TS15, *SeNHX1* transgenic tobacco line; 0, WT and TS15 under normal conditions; 0 + ATP/PP_i, WT and TS15 supplied with 1.5 mM ATP or PP_i under normal conditions; 200, WT and TS15 with 200 mM NaCl treatment; 200 + ATP/PP_i, WT and TS15 supplied with 1.5 mM ATP or PP_i after 200 mM NaCl treatment. Same letter on the bar top indicates that the values are not significantly different at $P \leqslant 0.05$ by LSD.

Table 1
Comparison of activities of energy metabolism enzymes by pH fluorescence quenching and vacuolar ΔH^+ flux using NMT in WT and TS15 plants under normal and 200 mM NaCl shock conditions.

Genotype	NaCl (mM)	Activities of energy metabolism enzymes (μmol P_i mg^{-1} protein h^{-1})		ΔH^+ flux (pmol cm^{-2} s^{-1})	
		V-ATPase	PPase	1.5 mM ATP	1.5 mM PP_i
WT	0	18.72 ± 0.65 a	17.47 ± 1.87 a	0.076 ± 0.012 b	0.017 ± 0.003 a
	200	20.93 ± 3.25 a	19.43 ± 1.21 a	0.560 ± 0.078 c	0.410 ± 0.054 c
TS15	0	18.86 ± 2.61 a	18.47 ± 1.84 a	0.083 ± 0.026 b	0.021 ± 0.008 a
	200	38.87 ± 2.12 b	20.65 ± 2.75 a	0.900 ± 0.099 d	0.482 ± 0.033 c

WT, wild-type tobacco plant; TS15, *SeNHX1* transgenic tobacco line. Vacuole located energy metabolism enzymes are represented by V-ATPase and PPase. Values are means ± SE ($n = 6$). Values with different letters in each line are significantly different at $P \leqslant 0.05$ by LSD test.

plants with or without 200 mM NaCl treatment. As shown in Table 1, under normal conditions, WT and TS15 plants exhibited comparable ΔH^+ flux after ATP (0.076 ± 0.012 and 0.083 ± 0.026 pmol cm^{-2} s^{-1}) or PP_i (0.017 ± 0.003 and 0.021 ± 0.008 pmol cm^{-2} s^{-1}) supply. Also, under 200 mM NaCl stress, there was no difference of vacuolar ΔH^+ flux between WT and transgenic plants after PP_i supply (0.410 ± 0.054 and 0.482 ± 0.033 pmol cm^{-2} s^{-1}). However, the ΔH^+ fluxes after ATP supply in transgenic plants (0.900 ± 0.099 pmol cm^{-2} s^{-1}) were significantly higher than those in WT plants (0.560 ± 0.078 pmol cm^{-2} s^{-1}). Therefore, the deduced activities of V-ATPase with 200 mM NaCl treatment were higher in transgenic than in WT plants.

To validate this conclusion, we also measured the activities of V-ATPase and PPase using the H^+ transportation assay described by Vera-Estrella et al. [1]. The result also showed that transgenic plants exhibited higher activities of V-ATPase than WT under 200 mM NaCl stress, whereas there were comparable activities of ATPase and PPase between transgenic and WT plants in other cases (Table 1). This was consistent with our results conducted by vacuolar ΔH^+ flux detection using NMT. Also, previously, it was confirmed that the enhanced salt tolerance of the transgenic plants was closely related to the higher activity of V-ATPase [9], and research in halophyte *Suaeda salsa* revealed that V-ATPase activity contributes more to enhanced salt stress while PPase plays a minor role [10].

Besides as an alternative method for measurement of the activities of V-ATPase and PPase, NMT for vacuolar H^+ flux detection revealed the dynamic process of H^+ flux with different treatments (Fig. 1). In addition, the enhanced activities of NHX1 in transgenic plants were directly indicated by net H^+ effluxes into cytoplast which increased after 200 mM NaCl shock (Fig. 1c and d). Especially, as shown in Fig. 1a–d, NMT can distinguish the slight difference of H^+ flux between transgenic and WT plants under normal conditions. These results are important for functional analysis of *SeNHX1*, which is beyond the capability of H^+ transportation assay with fluorescence quenching [1].

In summary, the suitability and accuracy of vacuolar H^+ flux detection with the modified NMT system were demonstrated in the present study. This simplified method with a vacuole isolation protocol and H^+ flux detection techniques can be widely used as an analytical tool for different kinds of ion flux detection at subcellular and organellar levels.

Acknowledgment

This work was supported by the Research Program from the Chinese Ministry of Agriculture (Grant 2009ZX08009-101B) and the Knowledge Innovation Project of Chinese Academy of Sciences (Grant No. KSCX2-EW-J-1).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.07.018.

References

[1] R. Vera-Estrella, B.J. Barkla, L. García-Ramírez, O. Pantoja, Salt stress in *Thellungiella halophila* activates Na^+ transport mechanisms required for salinity tolerance, Plant Physiol. 9 (2005) 1507–1517.
[2] W.M. Kuhtreiber, L.F. Jaffe, Detection of extracellular calcium gradients with a calcium-specific vibrating electrode, J. Cell Biol. 110 (1990) 1565–1573.
[3] Y. Xu, T. Sun, L.P. Yin, Application of non-invasive microsensing system to simultaneously measure both H^+ and O^{-2} fluxes around the pollen tube, J. Integr. Plant Biol. 48 (2006) 823–831.
[4] V. Perez, T. Wherrett, S. Shabala, J. Muniz, O. Dobrovinskaya, I. Pottosin, Homeostatic control of slow vacuolar channels by luminal cations and evaluation of the channel-mediated tonoplast Ca^{2+} fluxes in situ, J. Exp. Bot. 59 (2008) 3845–3855.
[5] I.A. Newman, Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function, Plant Cell Environ. 24 (2001) 1–14.
[6] I. Arif, I.A. Newman, N. Keenlyside, Proton flux measurements from tissues in buffered solution, Plant Cell Environ. 18 (1995) 1319–1324.
[7] D.M. Porterfield, E.S. McLamore, M.K. Banks, Microsensor technology for measuring H^+ flux in buffered media, Sensor. Actuat. B 136 (2009) 383–387.
[8] S.F. Zhou, X.Y. Chen, X.G. Zhang, Y.X. Li, Improved salt tolerance in tobacco plants by co-transformation of a betaine synthesis gene *BADH* and a vacuolar Na^+ /H^+ antiporter gene *SeNHX1*, Biotechnol. Lett. 30 (2008) 369–376.
[9] S.F. Zhou, Z.M. Zhang, Q.L. Tang, H. Lan, Y.X. Li, P. Luo, Enhanced V-ATPase activity contributes to the improved salt tolerance of transgenic tobacco plants overexpressing vacuolar Na^+ /H^+ antiporter *AtNHX1*, Biotechnol. Lett. 33 (2011) 375–380.
[10] B.S. Wang, L. Ulrich, R. Rafael, Effects of salt treatment and osmotic stress on V-ATPase and V-PPase in leaves of the halophyte *Suaeda salsa*, J. Exp. Bot. 52 (2001) 2355–2365.