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NaCl–induced Proteomes of Tonoplast from the Leaves of a Halophyte *Mesembryanthemum crystallinum* L.

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This study aims to examine the characteristics of vacuole ATPase (V–ATPase) activities and protein expression in vacuole fractions isolated from the common ice plant (*Mesembryanthemum crystallinum* L.) grown under control and salt–stressed conditions, using various centrifugation conditions of sucrose density gradient. The protein samples were analyzed using two–dimensional electrophoresis (2DE) gels. The results reveal that the vacuolar fractions collected at 20%, 30%, and 40% (w/w) sucrose density in the 0 mM, 100 mM, and 400 mM NaCl–stressed plants, respectively, exhibited the highest V–ATPase activities. Using the differential display with 2DE gels, the proteins in the vacuole fractions were analyzed, and the abundant proteins on each gel were identified based on their isoelectric points and molecular weights. The Arabidopsis database and the ExPASy–TagIdent tool were used to estimate the proteins. The results show 165, 194, and 199 abundant proteins with 41, 51, and 53 different proteins relating to the vacuole fractions among control samples (0 mM NaCl) and salt–stressed samples at 100 mM NaCl and 400 mM NaCl, respectively. Although the proteins on each 2DE gel varied among the samples, they were generally focused on ten physiological function groups, including pump proteins, transporter proteins, metal channel proteins, peptide transport proteins, channel proteins, stress response proteins, protease proteins, autophagic proteins, stress response proteins, and unknown proteins.

Key words: Common ice plant, Proteome, Salt stress, Tonoplast

INTRODUCTION

The vacuole plays a crucial role in plant growth and development, serving a multitude of functions such as storage and transport, intracellular environmental stability, and response to adverse stress environments. Plant cells possess up to 10,000 different proteins, yet vacuolar–located proteins account for only a fraction (1%) of the plant's total proteins (Martinoia *et al*., 2018). In contrast to other organelles, such as mitochondria and chloroplasts, the isolation of plant vacuoles via traditional methods is challenging and often results in fragile vacuoles (Robert *et al*., 2007). Hence, large–scale isolation and identification of vacuoles, as well as the qualitative and quantitative analysis of subcellular pathways, will greatly enhance our understanding of the plant system (Tan *et al*., 2019).

The common ice plant serves as an excellent model species for examining the functional genomics, metabolomics, and proteomics of crassulacean acid metabolism (CAM) plants. When grown under non–stressed conditions, this species exhibits C3 photosynthesis and completes its entire life cycle without displaying net nocturnal CO_2 uptake. However, under salinity or drought stress conditions, plants exhibit the physiological features of CAM plants (Cushman *et al*., 2008). CAM is induced under salt stress, leading to the accumulation of malic acid and NaCl in vacuoles during the night. The uptake of malate into the vacuole follows the electrochemical gradient of H^+ between the inner and outer vacuole membrane. Therefore, vacuole ATPase activity, which establishes the H^+ gradient, is increased at night and decreased during the day. Conversely, Na^+ is also taken up into the vacuole using the H^+ gradient created by the vacuole membrane ATPase, highlighting the need to maintain ATPase activity high during both day and night to prevent salt absorption (Epimashko *et al*., 2004, 2006). The vacuole membrane is expected to host various H^+ requiring reactions simultaneously under salt stress–induced CAM conditions. In the ice plant, under CAM conditions, stomata close during the day to minimize water loss, whereas they open at night to uptake CO_2. The CO_2 converts to malic acid, which is metabolized during the day, helping the plant to release CO_2 and use it for photosynthesis, a fundamental difference between the CO_2 fixation mechanisms of CAM, C3, and C4 plants.

This unique mechanism allows CAM plants to adapt and survive in arid lands with soil salinity. The induction of CAM is reported to change the molecular weight of vacuole membrane proteins (Bremberger and Lüttge, 1992). Interestingly, the ice plant possesses two types of vacuoles with contrasting functions, salt storing and malate cycling, which can co–exist in the same cells of leaves (Epimashko *et al*., 2004). Although the study provided experimental evidence of the physiological roles of the two separate vacuoles in the same ice plant cell, little is known about the protein characteristics and their functions on each vacuole under salt stress conditions via proteome analysis with the 2DE technique.

In recent years, proteomic technologies based on 2DE analysis have been increasingly applied to the study

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of plant organelle proteins, such as chloroplasts and mitochondria, in *Arabidopsis thaliana* and many other C3 and C4 photosynthesis species. However, successful application of 2DE analysis to plant vacuoles remains rare. For example, two proteomic analyses of the tonoplast used a membrane fraction isolated by sucrose density gradient and chromatographic separation, as reported by Sazuka *et al.* (2004) and Szponarski *et al.* (2004), but their results showed significant contamination from other membranes (Tan *et al.*, 2019). On the other hand, previous researchers have reported the isolation of intact vacuoles and proteomic analysis from suspension–cultured cells or vacuoles of *A. thaliana*, including Shimaoka *et al.* (2004), Jaquinod *et al.* (2007), Carter *et al.* (2004), and Ohnishi *et al.* (2018). However, most of these analyses have used SDS–PAGE and Western blot or LC–MS/MS, but not 2DE techniques for vacuolar proteome analysis. In previous work, we successfully applied the 2DE technique for tonoplast proteome analysis of two CAM species, *Kalanchoe pinata* and *Ananas comosus* (Lin *et al.*, 2008), as well as simultaneous chloroplast and mitochondria proteome analysis from the leaves of the ice plant under well–watered and drought–stressed conditions (Hong *et al.*, 2019).

In this study, we report the characteristics of V–ATPase activity and 2DE proteomic analysis of ice plant control samples (0 mM NaCl) and salt–stressed samples treated with 100 mM NaCl and 400 mM NaCl. We isolated and purified the ice plant vacuolar fractions from leaf samples with varying concentrations of sucrose to obtain the highest ATPase activities. We then measured vacuolar–ATPase activities and conducted one–dimensional SDS–PAGE analysis together with 2DE analysis on highly intact vacuoles. We identified differences in protein expression on each 2DE map under control and salt stress conditions. Finally, we present a detailed analysis of these proteins and their roles in each vacuole's accumulation of Na^+ and malate in the same cell of ice plant under salt stress conditions.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of the wild–type of the common ice plant were germinated according to the methods described by Sunagawa *et al.* (2007). The seeds were sterilized in 2.0% sodium hypochlorite solution for 7 min. After sterilization, seeds were put onto a germination medium (pH 5.7) containing 4.6 g L^{-1} MS salts (Murashige and Skoog, 1962), 1 × B5 vitamins (Gamborg *et al.*, 1968), 30 g/L sucrose and 10 g/L agar. Seedlings were grown in a growth chamber at 25ºC with 16h/8h (light/dark) photoperiod under a cool–white fluorescent light (70–80 μmol m^{-2} s^{-1}). The plants were grown hydroponically in a greenhouse under natural sunlight, oscillating between around 20ºC to 30ºC. When plants reached the growth stage at which the 4^{th}–6^{th} leaf appeared, NaCl was added to the culture solution to a final concentration of 0 mM, 100 mM and 400 mM as salt–stress treatment, and the pH was maintained at around 5.75.

Sample collection

To ensure the grow–up time was enough for ice plant to fully convert to CAM plants, approximately 0.5 g of each leaf sample was used to determine the pH value. The leaf samples were collected at 10 a.m., washed, dried, and finely ground with a mortar and pestle. Then, the pH of the leaf extract was measured with pH test paper (pH 0–14) (Whatman). Most ice plant treated with 400 mM NaCl became CAM plants after at least 14 days of salt treatment and their leaf extract solution showed pH less than 5, while the control and 100 mM NaCl treated samples were still C3 plants with a high pH value. Based on this test, the leaf of the control samples and salt–treated samples were collected after 14 days of NaCl treatment.

Vacuolar membrane fractionation/ Subcellular fractionation

Vacuolar fractionation for proteome analysis and ATPase determination were assayed according to Lin *et al.* (2008) and Epimashko *et al.* (2006) with a slight modification. Approximately 60 g of the ice plant's mature leaves were used for vacuolar membrane fractionation. After removing the main veins, leaf tissue was homogenized with 100 ml of buffer 1 containing 400 mM mannitol, 3 mM $MgSO_4.7H_2O$, 10 mM EGTA, 1 mM DTT, 1% (w/v) PVP–40, 100 mM tricin, 1 mM PMSF, 2 μg/ml leupeptin and 50 mM Tris – KOH (pH 8.0). After filtration through two layers of sterile Miracloth, the homogenate was centrifuged at 8,000 g for 15 min at 4°C (Rotor TA–7, Suprema 21, Tommy Seiko). The supernatant was centrifuged at 80,000 g for 50 min at 4°C (Rotor P70AT, CP75β, HITACHI Koki Co., Ltd.). After centrifugation, the supernatant was discarded and the pellet was suspended in 1 ml buffer 2 containing 400 mM mannitol, 10% (v/v) glycerol, 6 mM Tris/Mes (pH 8.0), 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, and layered onto 15 ml of sucrose gradient [(20%, 25%, 30%, and 35% (w/w)] for the control sample (0 mM NaCl), and 100 mM NaCl treated samples or 25%, 30%, 35% and 40% (w/w) sucrose gradient medium for 400 mM NaCl treated samples, and centrifuged in a swing–out–rotor at 90,000 g for 16 hours at 4° (Rotor P70AT, CP75β, HITACHI Koki Co., Ltd.). After centrifugation, about 1 ml of gradient fractions were collected and transferred to a new centrifuge tube, diluted with buffer 2, and centrifuged at 100,000 g for 30 min at 4°C.

The resulting precipitate was divided into two parts: one was dissolved in 1 ml of buffer 2 and used as vacuolar membrane collection for measuring vacuolar membrane ATPase activity, and the other was dissolved in 1 ml total protein extraction buffer of 2% (v/v) TritonX–100, 1% (w/v) PVP–40, 4 mM $MgCl_2$, 500 mM Tris/HCl (pH 8.3), 2% (v/v) β–mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin. The samples were frozen in liquid nitrogen and stored at –80°C for protein measurement and 2DE analysis. The protein content was measured with Bio–Rad Protein Assay Reagent (Bio–Rad)

using bovine serum albumin as the standard. The SDS–PAGE was performed using a LAPITAS mini–slab gel electrophoresis tank (Ato Corporation) according to the manufacturer's instruction. SDS–PAGE standards Low Range (Bio–Rad) was used as a size marker.

Analysis of vacuole ATPase

Vacuolar ATPase (V–ATPase) activity was assayed at 350 nm, room temperature in a reaction mixture containing 50 mM Tris/MES (pH 8.0), 0.02% (w/v) TritonX–100, 50 mM KCl, 1 mM Na_2MoO_4. $2H_2O$, 3 mM $MgSO_4$. $7H_2O$ and 1 mM ATP. In addition, 50 mM potassium nitrate, 40 nM bafilomycin and 0.25 mM ammonium vanadate were supplemented in the reaction mixture as the inhibitors of vacuolar membrane and mitochondrial ATPase, vacuolar membrane ATPase and plasma membrane ATPase, respectively. The reaction started with adding the sample and stopped with adding 1% (w/v) sodium dodecyl sulphate (SDS). The released isoelectric point (pI) from the substrate was determined in a reaction solution of 40.5 mM NH_4MoO_4, 0.01% (v/v) ammonia water 28%, 1 mM NH_4VO_3, 3.761% (v/v) HNO_3. The salts and sugars that inhibit the separation of proteins from the fractionated vacuolar membrane solution were assayed according to Kirch *et al*. (2000).

The reaction started with the addition of the sample and stopped with the addition of 1% (w/v) sodium dodecyl sulfate (SDS). The released Pi (phosphoric inorganic)

Two–dimensional gel electrophoresis

For vacuolar protein separation, 2DE was employed, and Decyder v7.0 software was used to quantify the abundance of different protein spot abundances on the 2DE gels. Vacuole proteins were resuspended in an IEF sample buffer consisting of 6 M urea, 2M thiourea, 2% (w/v) CHAPS, 0.0025% (v/v) bromophenol blue, 60 mM DTT, 0.5% (v/v) IPG buffer (pH 3–10 NL), 2 mM tributylphosphine and used immediately for isoelectric focusing with a 24 cm 3–10 immobilized pH gradient (IPG) strip (GE Healthcare, Inc., Pittsburgh, PA). Aliquots of 360 μL were used to rehydrate the dried leaves to 180 mm with a pH range of 3 to 10 nonlinear immobilized pH gradient strips for 13 hours. The IEF was performed for 1 min at 500 V, 1 1/2 hr at 3500 V gradient, and then held at 3500 V for 6 hr 20 min at 20°C on a flat–bed electrophoresis unit (Multiphor Ⅱ system, Amersham Pharmacia Biotech, USA). Immobilized pH gradient strips were then transferred to 15 mL of an equilibration buffer consisting of 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) BPB and 1% (w/v) DTT for the first equilibration step and 2.5% (w/v) iodoacetamide for the second equilibration step. The strips were incubated for 15 min with vibration. The equilibrated strips were slotted at 15°C into an ExcelGel SDS XL Gradient 12–14, which performed for 35 min at 600 V, 20 mA of the first steps, and 1 1/2 hour at 600 V, 50 mA of the second step.

Different protein detection among the gels

The vacuolar proteins separated on 2DE gels were fixed for 30 min in 7% (v/v) methanol with 10% (v/v) acetic acid, washed three times with distilled water, and stained for at least 3 h with Sypro Ruby (Invitrogen, Inc., Carlsbad, CA). The separated proteins on the 2DE gels were stained with Sypro Ruby (Bio–Rad, USA). The protein spots were detected and characterized by using a Typhoon 9000E (Amersham Bioscience Corp., USA) scanner. The protein expressions in the 2DE maps were probed by using Image Master 2DE software (Amersham Pharmacia Biotech, USA). Molecular weight and Pi standards from Amersham Pharmacia Biotech were used to confirm fixed pH gradient positioning on first dimensional separation and to identify the apparent molecular mass on the second dimensional separation. Tool was used for the homology search based on the isoelectric point (pI) and molecular weight (Mw), which was estimated within 5% error (http://tw.expasy.org/tools/tagIdent.html) based on the Arabidopsis database. The pI and molecular weight (Mr) standards were used to confirm the fixed pH gradient positioning in the first dimensional separation and identify the apparent molecular mass in the second dimensional separation, respectively. Three replicate gels were run to obtain vacuolar protein maps for each sample.

RESULT AND DISCUSSION

The specific ATPase activity in the ice plant vacuolar membrane fractions

By previous research, which indicated functional V–ATPase activity in both light (<35%) and heavy (>35%) sucrose (w/w) membrane fractions of NaCl–stressed ice plant (Epimashko *et al*., 2006), this study utilized fractionated light vacuolar membrane fractions of 0 mM NaCl (control) and 100 mM NaCl–stressed ice plant at 20–35% (w/w) sucrose, as well as fractionated heavy vacuolar membrane fractions of 400 mM NaCl–stressed samples at 20–40% (w/w) sucrose, to measure vacuolar membrane ATPase (V–ATPase) activity and assess its purity and intactness.

In addition, the activity of V–ATPase can be distinguished from other ATP–hydrolyzing proton pumps by its sensitivity to specific inhibitors (Sze, 1985). Vanadate (VO_3) is a potent inhibitor of certain plasma membrane ATPases but does not affect other ATPases, such as endoplasmic reticulum Ca_2^+–ATPase and mitochondrial ATPase (Luo *et al*., 2000). Nitrate (NO_3) is known to inhibit V–ATPase and F–ATPase in mitochondrial or chloroplast membranes (Lüttge and Ratajczak, 1997). Furthermore, bafilomycin A1 is a strong inhibitor of vacuolar–type H^+–ATPase in vitro, while this antibiot does not affect other type ATPases, such as F_1 and F_o–ATPase (Bowman *et al*., 1988). Therefore, these inhibitors can serve as indicators of vacuolar membrane fraction purity and intactness.

Fig. 1 illustrates the ATPase activity of vacuolar membrane fractions detected at the light sucrose density range of 20–35% (w/w) from the control ice plant in the

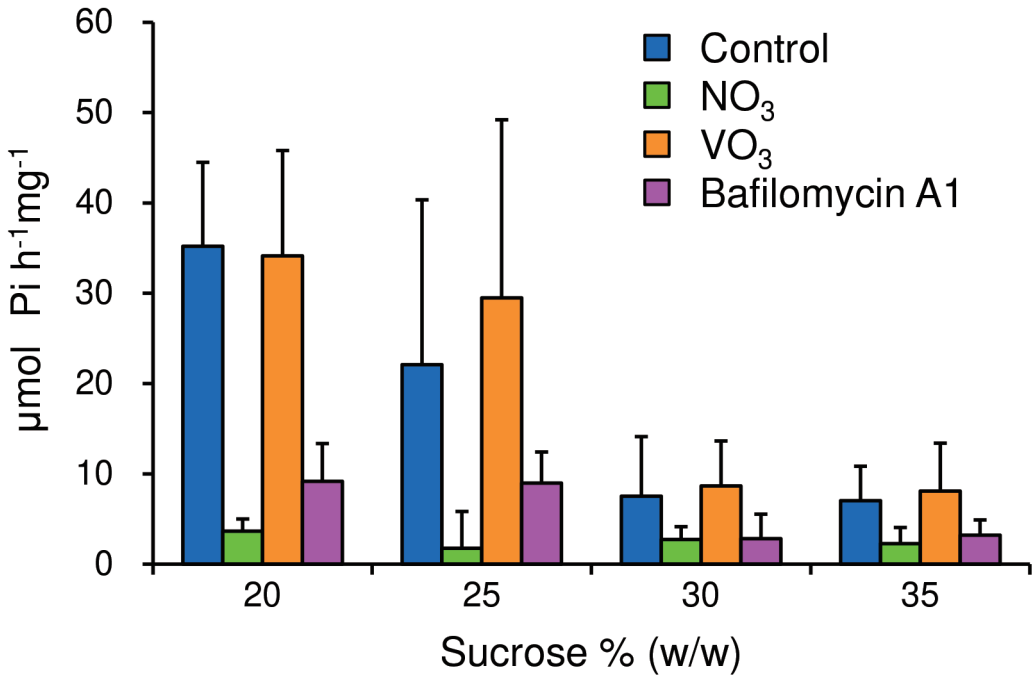

Fig. 1. V–ATPase activities in the membrane regions at sucrose density of 25–35% (w/w) in the presence and absence of 25 mM VO_3, 50 mM NO_3, or 40 mM bafilomycin A1 of the ice plant control samples (0 mM NaCl). Values represent mean a standard error, n=3.

presence and absence of inhibitors. The V–ATPase was assessed by quantifying the amount of inorganic phosphate released by 1 mg of vacuolar membrane protein upon degradation of ATP within 1 hour. The data indicates that V–ATPase activities in each membrane fraction were significantly different across varying sucrose density gradients. Vacuolar membranes were fractionated from 0 mM NaCl–treated leaf blades at sucrose densities of 20% to 35% (w/w), and the activity of ATPase was measured. VO_3 is a potent inhibitor of certain plasma membrane ATPases; however, the value obtained in the presence of VO_3 was found to be similar to that of the control without the inhibitor. The high purity of the isolated membranes is evident from the data. The values obtained without the addition of ATPase inhibitor were similar to those obtained with VO_3, which suggests that VO_3 is not inhibiting the vacuolar membrane ATPase. The values of the vacuolar membrane and mitochondria treated with NO_3 (nitrate), an inhibitor of ATPase activity, and BafilomycinA (bafilomycin), a specific inhibitor of the vacuolar membrane, were similar. This suggests that there is minimal mitochondrial contamination. The highest ATPase activity was observed at a sucrose concentration of 20% (w/w) in the density gradient. These results indicate that the membrane region at the sucrose density of 20% (w/w) is suitable for SDS and proteomic analysis using the 2DE technique.

Fig. 2 shows the ATPase activity of vacuolar membrane fractions isolated from the ice plant subjected to 100 mM NaCl stress, as detected at the light sucrose density of 20–35% (w/w) in the presence and absence of inhibitors. Notably, the V–ATPase activities in the membrane regions lacking inhibitors were markedly higher than those in the control sample. The V–ATPase activities in the vacuolar membrane regions at sucrose densities of 20%, 25%, 30%, and 35% (w/w) were significantly decreased. High purity of the vacuolar membrane was confirmed, indicating less contamination of mitochondria and cell membrane. The highest V–ATPase activity without inhibitor was observed in the 20% (w/w) sucrose gradient region of the 100 mM NaCl treated sample. While the V–ATPase activity in the 30% (w/w) sucrose region was relatively lower than that in the 20% (w/w) sucrose region, it was still much higher than the regions at 25% and 35% (w/w) sucrose. The V–ATPase activity of the 30% (w/w) sucrose region in the presence of 50 mM NO_3 or 40 mM bafilomycin A1 was very low. In contrast, the activity with 25 mM VO_3 was similar to that without the inhibitor. These results suggest that the 30% (w/w) sucrose gradient solution is less contaminated with other membrane ATPases and is considered the best for this treatment.

Fig. 3 demonstrate the V–ATPase activity in membrane regions, without inhibitors, at sucrose densities ranging from 25% to 40% (w/w) isolated from 400 mM NaCl–stressed plants. In the 400 mM NaCl–treated samples, the V–ATPase activity was highest in the membrane region at 40% (w/w) sucrose, followed by those at 25%, 30%, and 35% (w/w) sucrose, respectively. The V–ATPase activity of the regions at 40% (w/w) sucrose with 25 mM VO_3 was slightly lower than those without VO_3. In addition, the V–ATPase activity with 50 mM NO_3 or 40 mM bafilomycin A1 was very low in this region compared to other regions at 25%, 30%, and 35% (w/w) sucrose. These results suggest that the vacuolar membrane region at 40% (w/w) sucrose from 400 mM NaCl–

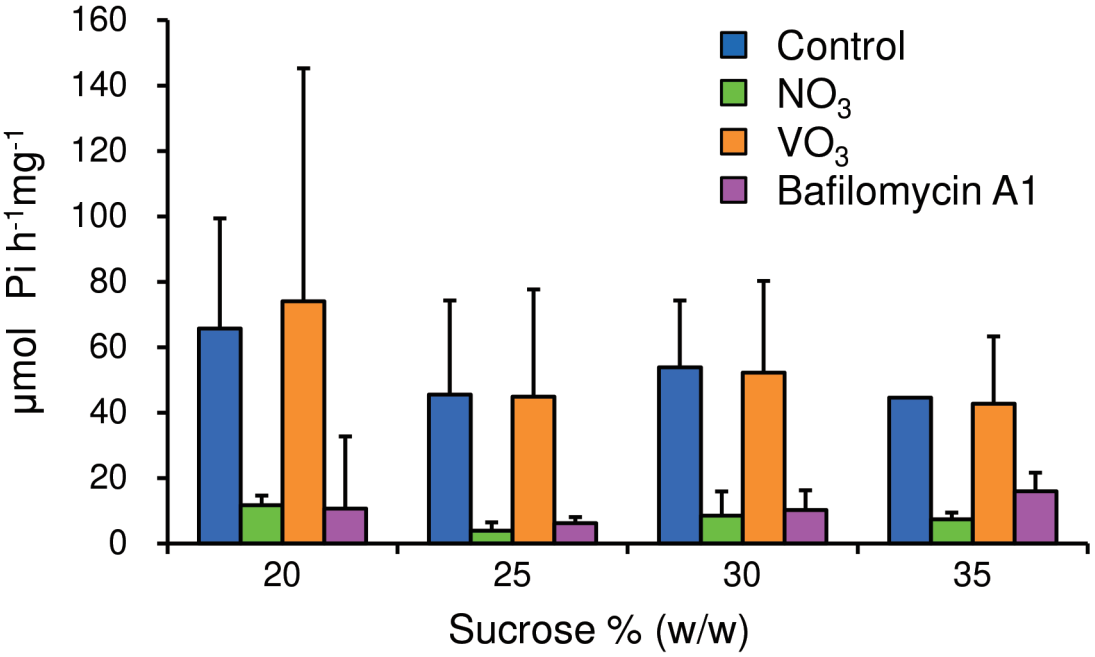

Fig. 2. V–ATPase activities in the membrane regions at the sucrose density of 25–35% (w/w) in the presence and absence of 25 mM VO_3, 50 mM NO_3, or 40 mM bafilomycin A1 of the 100 mM NaCl stressed samples. Values represent mean standard error, n=3.

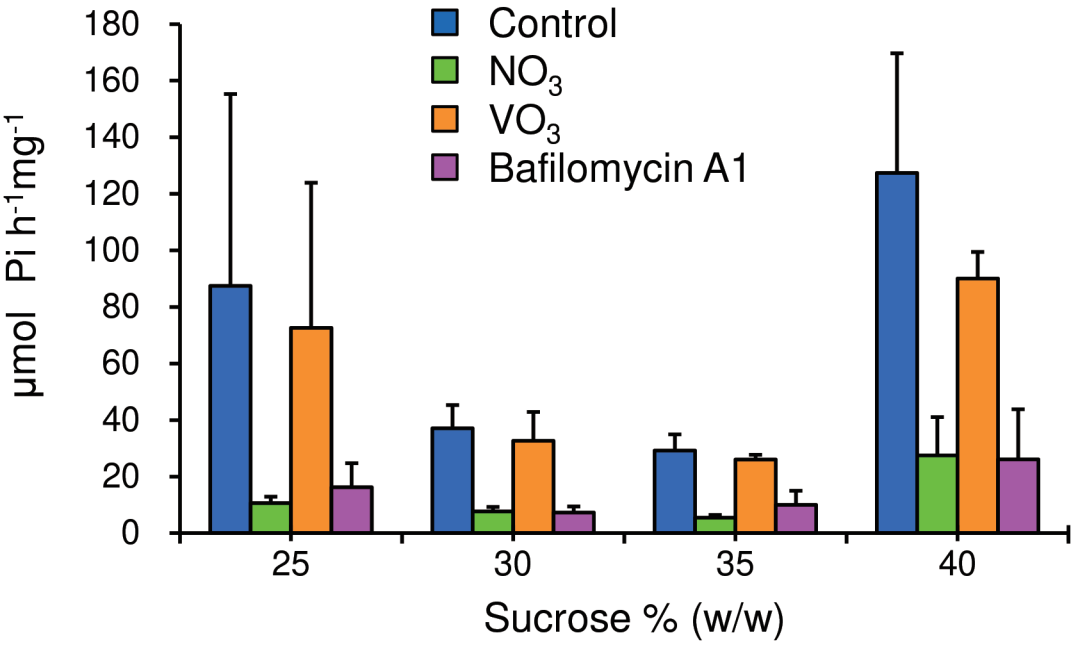

Fig. 3. V–ATPase activities in the fractions of different sucrose densities from the 400 mM treated sample in the presence and absence of VO_3, NO_3 and bafilomycinA1 inhibitors. Values represent mean standard error, n=3.

stressed plants was less contaminated with other membrane ATPases. The V–ATPase activity in this region exhibited a high level. These findings, in conjunction with those presented in Fig. 1 and Fig. 2, suggest that the vacuolar membrane regions at 20%, 30%, and 40% (w/w) sucrose of control and the salt–treated samples at 100 mM NaCl and 400 mM NaCl can be utilized.

Fig. 4 depicts the differential expressions observed on SDS–PAGE gels of the vacuolar membrane protein isolated from the control sample (0 mM NaCl) and salt–stressed samples treated at 100 mM NaCl and 400 mM NaCl. The results indicate that most of the vacuolar protein was found within the 30–70 kDa range, while some new protein bands, such as bands 1 and 2, appeared in the 100 mM NaCl salt–stressed sample. Conversely, bands 3, 4, 5, and 6 showed reduced expression in both

the 100 mM NaCl and 400 mM NaCl samples. Fig. 4 displays the SDS–PAGE expression of vacuolar proteins isolated from salt–treated samples at 0 mM NaCl, 100 mM NaCl, and 400 mM NaCl, using 20%, 30%, and 40% (w/w) sucrose in vacuole isolation, respectively.

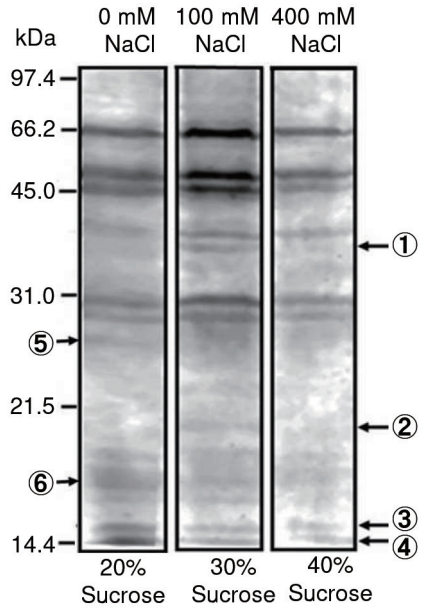

Fig. 4. SDS–PAGE expression of vacuole proteins isolated from salt treated samples at 0 mM NaCl, 100 mM NaCl and 400 mM NaCl using 20%, 30% and 40% (w/w) sucrose in vacuole isolation, respectively. 1, 2: New protein bands were detected on 100 mM NaCl sample. 3, 4, 5, and 6: Protein bands were reduced on 100 mM NaCl and 400 mM NaCl samples.

Vacuole membrane Protein separation in 2 DE gels for proteomic analysis

Cosentino *et al*. (2013) conducted a proteomic analysis of leaf microsomal fractions from the ice plant under salt–stressed treatment. However, they employed a non–gel technique known as MudPIT (Multidimensional Protein Identification Technology) for separating and identifying individual components of complex protein and peptide mixtures from salt–stressed ice plant leaves. In the present study, we used the 2DE procedure to conduct a proteomic survey of the ice plant's vacuolar membrane–associated proteins. It is widely acknowledged that proper 2DE sample preparation is a fundamental aspect of proteomic approaches and can significantly impact the results of numerous analyses. Moreover, as proteomics research often involves various approaches (Hong *et al*., 2019), it was necessary to assess the suitability of the isolated vacuolar membrane regions and their protein extraction for further proteome analyses of the ice plants under both control and salt–stressed conditions. In these experiments, samples were obtained from vacuolar membrane regions at 20%, 30%, and 40% (w/w) sucrose from the ice plants exposed to non salt–treated control and salt–treated samples at 100 mM NaCl and 400 mM NaCl, respectively, for the 2DE assay. Following the extraction and purification of vacuolar membrane proteins, the protein contents were quantified via the Bradford protein assay to determine the protein concentration of the vacuolar membrane samples in the rehydration buffer before transferring them to IPG strips in the 2DE assay.

Fig.s 5, 6, and 7 show the Sypro ruby–stained 2DE maps of vacuolar membrane region proteins at 20%, 30%, and 40% (w/w) sucrose of the salt–treated samples from the ice plants at 0 mM NaCl, 100 mM NaCl, and 400 mM NaCl, respectively. Protein spots in gels were detected using Image Master 2DE software (Amersham Pharmacia Biotech, USA).

A total of 165, 194, and 199 abundant proteins, consisting of 40, 50, and 52 different proteins, respectively, were found in the vacuole fractions among the control sample (0 mM NaCl) and salt–stressed samples at 100 mM NaCl and 400 mM NaCl. Among the vacuolar membrane regions at 20% (w/w) sucrose of the control sample (0 mM NaCl), 165 proteins were detected, with the majority located in a pH range of 4 to 9. The molecular masses of over 95% of these proteins ranged between 21.5 and 97.4 kDa, while less than 5% were detected between 21.5 and 14.4 kDa (Fig. 5).

Approximately 194 proteins were identified in the vacuolar membrane regions at 30% (w/w) sucrose of the 100 mM salt–stressed sample in the ice plants (Fig. 6). Comparing to the control sample, some protein spots disappeared, while others became more prominent and well–defined under 100 mM salt–stress conditions (Fig. 5 and Fig. 6).

Additionally, 199 abundant proteins were detected in the vacuolar membrane regions at 40% (w/w) sucrose of the 400 mM salt–stressed sample (Fig. 7). Most of the proteins detected ranged from 14.4 to 97.4 kDa. Compared to the control sample, the protein abundance in the 100 mM NaCl treated sample was not only much higher but also constituted a higher molecular mass (66.2 to 97.4 kDa) among the detected proteins (Fig. 5 and Fig. 7). These results suggest that most of the vacuolar membrane proteins extracted using this protocol were successfully transferred from strips to the 2DE gels. The overall quality of the proteins was superior for proteome analysis. Furthermore, the vacuolar membrane protein expression on each 2DE gel in Fig. 5, 6, and 7 was highly accurate. The isoelectric points (pI) and molecular masses (kDa) of vacuolar membrane samples from the ice plant at 0 mM NaCl, 100 mM NaCl, and 400 mM NaCl were compared to known data for isoelectric points and molecular masses of vacuolar proteins from Arabidopsis thaliana (Carter *et al*. 2004). The comparison is presented in Table 1. The results demonstrate that 41, 51, and 53 different proteins were found to be abundant in the vacuole fractions of the control sample, as well as salt–stressed samples at 100 mM NaCl and 400 mM NaCl, respectively. Although the presence of these proteins varied among each sample's 2DE gels, they generally fell into ten physiological function groups. Of the 59 proteins detected across the three samples, five were identified as H^+ pump–related proteins, ten as

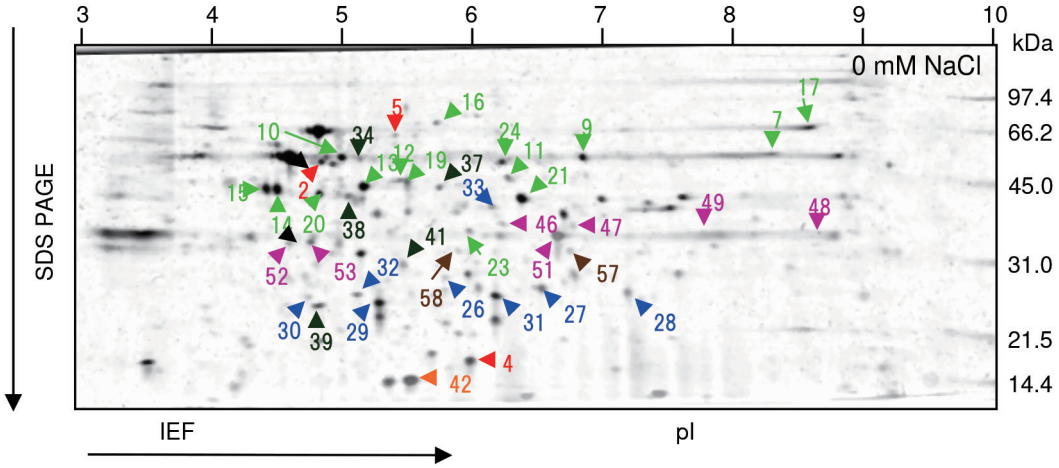

Fig. 5. Typical Sypro–Ruby® stained 2DE gels for the ice plant vacuolar proteins of 0 mM NaCl treated sample. Different protein expressions from the spots in this gel were labelled with different colors as below: Red color: H^+ pump–related protein, Green leaf color: transporter protein, Blue color: channel protein, Green color: Protease protein, Orange color: Autophagy protein, Purple color: stress response protein, Brown color: vesicular transport protein and unknown protein: Black color.

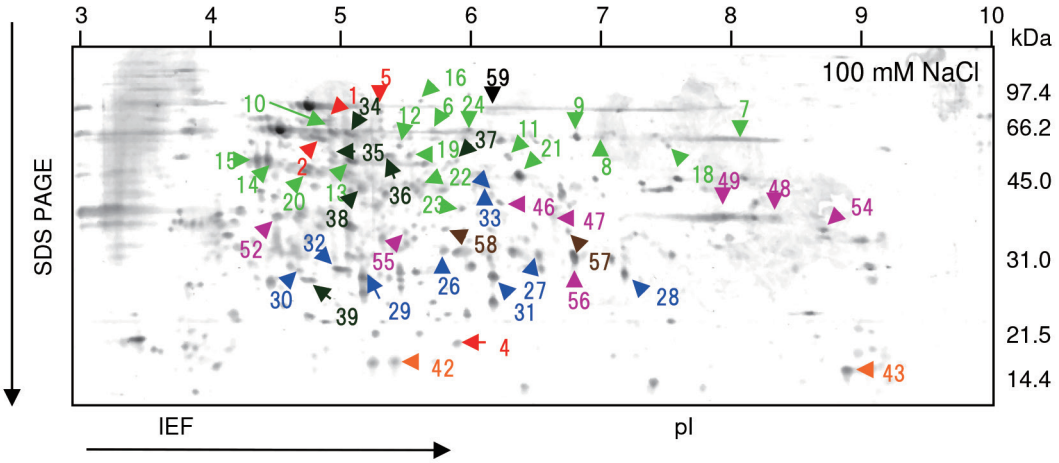

Fig. 6. Typical Sypro–Ruby® stained 2DE gels for the ice plant vacuolar proteins of 100 mM NaCl treated sample. Different protein expressions from the spots in this gel were labeled with different colors as shown in Fig. 5.

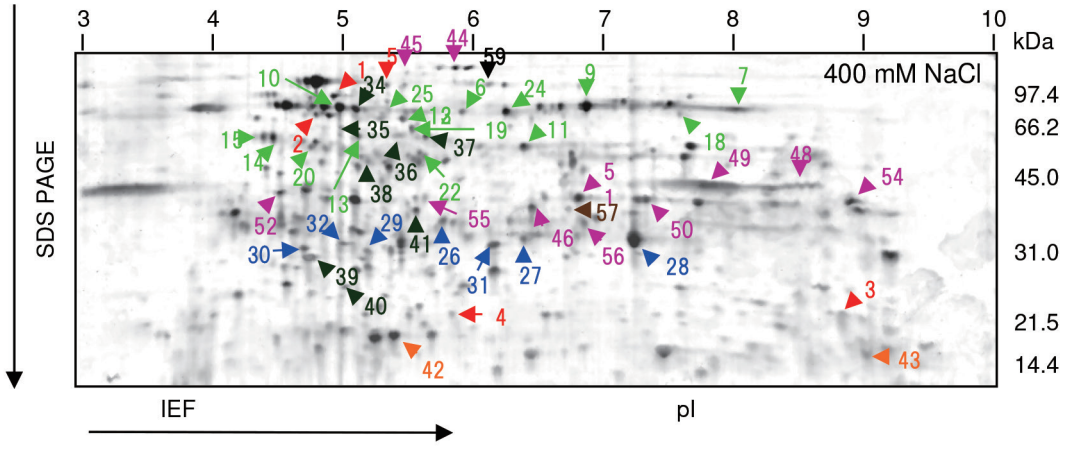

Fig. 7. Typical Sypro–Ruby® stained 2DE gels for the ice plant vacuolar proteins of 400 mM NaCl treated sample. Different protein expressions from the spots in this gel were labeled with different colors as shown in Fig. 5.

Table 1. Salt stress–induced tonoplast proteins from leaves of plants grown with 0 mM, 100 mM and 400 mM NaCl.

Protein	Expression intensity (%)			PI	Mw (Da)
	0 mM*	100 mM	400 mM		
Pump protein					
Vacuolar ATP synthase catalytic subunit A	–	26.6	70.2	5.1	68813
Vacuolar ATP synthase subunit B	59.8	37.8	53.3	5	54108
Vacuolar ATP synthase 16kDa proteolipid Subunit 1/3/5	–	–	27.1	8.6	16572
Putative vacuolar ATP synthase subunit F	63	22.3	29.7	6.1	14259
Pyrophosphatase energized vacuolar membrane proton pump1	20.6	24.1	39.4	5.1	80820
Transporter protein					
Na^+/H^+ antiporter 1	81.6	44.9	88.9	6.7	59514
Na^+/H^+ antiporter 3	–	18.1	–	6.9	55607
Na^+/H^+ antiporter 4	41.2	43.6	57.2	8.2	58872
Na^+/H^+ antiporter 5	86.1	41.6	84	5.1	56847
Na^+/H^+ antiporter 6	–	22.4	57.4	5.7	59339
Vacuolar cation/H^+ antiporter 2	89.5	47.3	56.4	4.7	48216
Vacuolar cation/H^+ antiporter 3	37.6	25	52.4	5.5	49852
Vacuolar cation/H^+ antiporter 4	38.6	22.4	18.8	6	48700
Vacuolar cation/H^+ antiporter 5	90.4	51	73.9	4.9	48096
Putative vacuolar cation/H^+ antiporter 6	93	53.1	54.4	5.2	45880
Metal ion transporter protein					
Cd/Zn transport ATPase 4	32	36.9	–	5.8	81985
Al tolerance related ATP binding cassette transporter	–	66.2	–	8.1	69104
Metal tolerance protein C2	51.7	25	46.5	5.8	43816
Metal tolerance protein C3	79.9	45.1	45.6	5.1	44053
Metal tolerance protein C4	–	19.6	37.4	7.3	50104
Zn transponrter ZAT–1	92.5	60.3	66.7	6.2	41028
Metal tolerance protein A2	–	19.6	37.8	5.8	41222
Putative metal tolerance protein A1	28.9	23.7	–	6	37345
Vacuolar malate transporters	66.7	34.5	80.7	6.3	58098
Peptide transporter					
Peptide transporter PTR2	–	–	23.5	5.5	64422
Channel protein					
Aquaporin TIP 1–1	75.9	49.4	70.3	6	25620
Aquaporin TIP 1–2	38.8	43.2	55.9	4.9	25849
Aquaporin TIP 2–3	83.2	47.2	39.7	5.2	25246
Aquaporin TIP 3–1	32.6	45.5	81.5	7.2	28308
Aquaporin TIP 4–1	15.2	26.4	50.4	5.8	20673
Putatime aquaporin TIP2–2	17.5	25.7	50.2	4.7	25080
Putative aquaporin TIP3–2	47.1	31.1	47.2	6.5	28183
Ca^{2+} dependent K^+ channel1	29.4	20.6	–	6.2	40727

Table 1. (continued)

Protein	Expression intensity (%)			PI	Mw (Da)
	0 mM*	100 mM	400 mM		
Stress response protein					
Phospholipase D α 1	–	–	14.1	5.5	91848
Phospholipase D α 2	–	–	64	5.8	91598
Peroxidase 12 (precursor)	19.1	24.2	48.2	8.4	36119
Peroxidase 17 (precursor)	47.9	32.4	64.3	5	34548
Peroxidase 22 (precursor)	–	17.7	25.9	5.7	35253
Peroxidase 23 (precursor)	–	34.9	68.3	8.5	35285
Peroxidase 32 (precursor)	18.2	26.2	59.4	6.1	35713
Peroxidase 33 (precursor)	–	–	58.3	7	35625
Peroxidase 34 (precursor)	29.1	63.6	74.9	7.7	35696
Peroxidase 37 (precursor)	48.5	–	71	7	35800
Peroxidase 38 (precursor)	62.6	31.1	–	7	35683
Peroxidase 54 (precursor)	7.8	31.2	60.7	4.5	34180
Basic endochitinase B (precursor)	28.8	53.8	37.9	7	31700
Protease protein					
Myrosinase (precursor)	37.6	40.8	74.2	5.4	59067
Vacuolar processing enzyme alpha–isozyme (precurso)	–	22.8	30.1	5.3	50620
Vacuolar processing enzyme gamma–isozymes (precurso)	–	30.8	28.7	5.4	51376
Serine carboxypeptidase like 8 (precursor)	38.4	31.3	60	5.8	47188
Xylem cysteine proteinase 2 (precursor)	44.4	31.8	35.2	5.2	39780
Thiol protease aleurone (precursor)	54.3	30	55.9	4.6	23423
Thiol protease aleurone like (precursor)	–	–	37.3	5.1	23654
Strictosidine synthase 1 (precursor)	28.5	–	54.8	5.6	32960
Autophasy protein					
Autophasy related protein 8g (precursor)	59	23.7	75.4	5.9	13937
Autophasy related protein 8f (precursor)	–	40.5	34.6	8.7	13762
Vecicle transport protein					
Syntaxin –21	17.9	20.4	22.9	6.9	31061
Syntaxin –22	14.2	20.8	22.9	6	29481
Unknown membrane protein At1g63010	–	15.6	31.9	–	–

*concentration of NaCl treated for plant culture.

transporter proteins, nine as metal channel proteins, one as a peptide transport protein, eight as channel proteins, thirteen as stress response proteins, eight as protease proteins, two as autophagy proteins, two as stress response proteins, and one as an unknown membrane protein At1g63010. Among the five pump proteins detected in the 400 mM NaCl treated sample, vacuolar ATP synthase 16kDa proteolipid subunit 1/3/5 was not detected in either the control or the 100 mM NaCl samples. The vacuolar ATP synthase catalytic subunit A was also detected in the 100 mM and 400 mM NaCl treated samples, but was not present in the control sample (Table 1).

Among transporter proteins, Na^+/H^+ antiporter 3 was detected only on the 100 mM NaCl treated sample, while it was not found on the control and 400 mM NaCl treated samples. In contrast, Na^+/H^+ antiporter 6 was detected on the control sample but not on the 100 mM NaCl and 400 mM NaCl treated samples. Nine metal channel proteins were detected on the 100 mM NaCl treated samples, while only six of this group were detected on the control and 400 mM NaCl treated samples. The ATP binding cassette transporter protein related to Al tolerance was detected only in the 100 mM NaCl treated samples and not in the control or 400 mM NaCl treated samples. Metal tolerance protein C4 and metal tolerance

protein A2 were detected in 100 mM and 400 mM NaCl treated samples but were absent in the control sample. Conversely, Cd/Zn transport ATPase 4 and putative metal tolerance protein A1 were detected in both the control and 100 mM NaCl treated samples but were absent in the 400 mM NaCl treated samples (Table 1). In the 400 mM NaCl treated samples, only one peptide transport protein, PTR2, was detected, whereas it was not found in either the control or 100 mM NaCl treated samples. Eight channel proteins were identified in almost all three samples, including the control, 100 mM, and 400 mM NaCl treated samples, except the Ca^{2+}–dependent K^+ channel 1, which was present in the control and 100 mM NaCl treated samples but not detected in the 400 mM NaCl treated sample. Among the stress response proteins, phospholipase Dα1, phospholipase Dα2, and peroxidase 33 (precursor) were found in both the control and 100 mM NaCl treated samples but were not observed in the 400 mM NaCl treated sample. Furthermore, peroxidase 2 (precursor) and 23 (precursor) were identified in both 100 mM and 400 mM NaCl treated samples, but not in the control sample. Peroxidase 37 (precursor) and peroxidase 38 (precursor) were exclusively detected on the control samples and not found on the 100 mM and 400 mM NaCl treated samples, respectively. Eight protease proteins were identified in the 400 mM NaCl treated samples, while only the 5 and 6 protease proteins were detected in the control and 400 mM NaCl samples, respectively. The autophagy–related protein 8f (precursor) was absent from the control sample, while two autophagy proteins were found in both control and salt–stressed samples. Two vesicle transport proteins, syntaxin–21 and syntaxin–31, were identified in all samples. Conversely, an unknown membrane protein, At1g63010 was detected in the 100 mM and 400 mM NaCl treated samples, but not in the control.

AUTHOR CONTRIBUTIONS

Hoang Thi Kim Hong contributed to manuscript preparation, critically reviewed, and provided suggestions and comments. Terutaka Nagai contributed to sample preparation, data collection, and analysis. Sakae Agarie designed the study, supervised data collection, and critically reviewed the manuscript with valuable suggestions and comments. All authors have read and approved the final manuscript.

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