Salt stress alters membrane lipid content and lipid biosynthesis pathways in the plasma membrane and tonoplast

Qi Guo, Lei Liu, Thusitha W.T. Rupasinghe, Ute Roessner and Bronwyn J. Barkla

1 Faculty of Science and Engineering, Southern Cross Plant Science, Southern Cross University, Lismore, NSW 2480, Australia
2 School of BioSciences, The University of Melbourne, Parkville 3010, Australia
3 Sciex, Mulgrave, VIC 3170, Australia

*Author for correspondence: bronwyn.barkla@scu.edu.au
†Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Bronwyn J. Barkla (bronwyn.barkla@scu.edu.au).

Abstract

Plant cell membranes are the sites of sensing and initiation of rapid responses to changing environmental factors including salinity stress. Understanding the mechanisms involved in membrane remodeling is important for studying salt tolerance in plants. This task remains challenging in complex tissue due to suboptimal subcellular membrane isolation techniques. Here, we capitalized on the use of a surface charge-based separation method, free flow electrophoresis, to isolate the tonoplast (TP) and plasma membrane (PM) from leaf tissue of the halophyte ice plant (Mesembryanthemum crystallinum L.). Results demonstrated a membrane-specific lipidomic remodeling in this plant under salt conditions, including an increased proportion of bilayer forming lipid phosphatidylcholine in the TP and an increase in nonbilayer forming and negatively charged lipids (phosphatidylethanolamine and phosphatidylserine) in the PM. Quantitative proteomics showed salt-induced changes in proteins involved in fatty acid synthesis and desaturation, glycerolipid, and sterol synthesis, as well as proteins involved in lipid signaling, binding, and trafficking. These results reveal an essential plant mechanism for membrane homeostasis wherein lipidome remodeling in response to salt stress contributes to maintaining the physiological function of individual subcellular compartments.

Introduction

Salts are an essential natural resource, but the degradation of agricultural land caused by excessive salts in the soil poses a substantial challenge to crop cultivation, leading to considerable economic losses and threatening global food security at a time of rapid population growth (Munns and Gilliham, 2015; Rojas et al., 2016; Corwin, 2020). The existence of halophytes (salt-tolerant plant species), and the different responses to salt stress observed between genotypes within glycophytes (salt-sensitive species), suggests that there must be a wide range of traits that can be exploited to help improve the salt tolerance of crops (Yamaguchi and Blumwald, 2005). However, efforts toward salt-tolerant crop breeding have not been widely successful as the fundamental mechanisms of stress tolerance in plants are complex, and many...
processes remain to be comprehensively understood (Hanin et al., 2016).

The similar sensitivities of cytoplasmic enzymes to salinity in both halophytes and glycophytes suggest that maintaining cellular ion homeostasis is the critical requirement for plants to survive high salt concentrations (Glenn et al., 1999). Therefore, studies into understanding the molecular mechanisms involved in salt tolerance have primarily focused on the regulation of sodium transport at the plasma membrane (PM) and tonoplast (TP, the vacuole membrane) in plants in response to salinity (Shabala and Munn, 2012; Yamaguchi et al., 2013; Barkla and Vera-Estrella, 2015; Ahmad et al., 2016; Mathias et al., 2017; Munn, et al., 2020). This important role of membrane transport also highlights the regulatory role of biological membranes in ionic and osmotic homeostasis maintenance through their role in sequestration and compartmentalization in salt-stressed plants.

Lipids are the principal constituents of biological membranes, with proteins embedded or attached directly through protein/protein or protein/lipid interactions to the lipid bilayer structure (Watson, 2015; Banfalvi, 2016; Harayama and Riezman, 2018; Cheng and Smith, 2019). The spatial partitioning provided by subcellular membranes within a cell creates distinct enclosed environments, allowing for proteins as well as other molecules to fulfill specific biological functions of individual organelles (Thul et al., 2017). Compositional features of these membranes, for example, specific lipid and protein constituents, are key aspects for defining organelle identity, transduction of signaling, recruitment of proteins, and maintenance of distinct ionic and pH gradients (van Meer et al., 2008; Okazaki and Saito, 2014; Simon et al., 2016; Plat and Jaillais, 2017; Barkla et al., 2018; Plat et al., 2018; Casares et al., 2019; Guo et al., 2019), which are vital when considering a plant’s response to abiotic stress.

Changes to membrane lipid composition in plants in response to salinity have been shown to alter membrane protein activity and membrane permeability for water, ions, and metabolites (Gamper and Shapiro, 2007; Tsydendambaev et al., 2013). However, the majority of the comparative studies have focused on bulked tissue membrane extractions, an approach that often masks organelle-specific changes (Sumner, 2010). The few studies that have focused on salt-induced changes to the membrane lipid composition of individual subcellular compartments have been limited to either chloroplasts or PM, with only a few specific lipid classes analyzed (Wu et al., 2005; Kodedova and Sychrova, 2015; Bejaoui et al., 2016; Omoto et al., 2016). Whereas the contribution of other membranes, such as the TP (the vacuole membrane), which plays a major role in ionic and osmotic adjustment during plant salt acclimation (Fukuda et al., 2004; Barkla et al., 2009), has been largely overlooked. Work in this area has been hampered by the limited techniques available to fractionate the subcellular membranes.

Free flow electrophoresis (FFE) is a technique that can exploit differences in the overall charges of bio-particles (proteins, organelles, and membranes), allowing for their separation in a liquid phase according to subtle charge differences, resulting in high-resolution fractionation (Roman and Brown, 1994; Eubel et al., 2007; Pamme, 2007; Turgeon and Bowser, 2009; Islinger et al., 2010; Eichacker et al., 2015; Barkla, 2018; Islinger et al., 2018). Our recent study using FFE combining mass spectrometry-based proteomic analysis allowed the simultaneous fractionation and profiling of multiple subcellular membranes from the leaf tissue of the ice plant based on specific marker protein identification (Guo et al., 2021). This study provided insight into the protein profile of subcellular compartments under salt stress in context to their biological role and gave a more comprehensive picture of the overall response of the cell to salinity.

In this study, using leaf microsomal membranes fractionated by FFE, we carried out a targeted lipidomic approach to quantitatively characterize the alteration in lipid composition of PM, TP, and a combined membrane sample of all FFE fractions (combined (Cmb)), between control and salt-treated ice plants. Results demonstrated membrane-specific differences in the lipid profiles of PM, TP, and Cmb under control conditions and provided insight into the organelle-specific membrane lipid regulation in plant cells in response to salinity. Reducing the complexity of the membrane sample by FFE helped improve the coverage of peptide identifications and facilitated the detection of a high number of proteins using the in-house generated spectral library for Sequential Windows Acquisition of All Theoretical Mass Spectra (SWATH-MS) quantitative proteomic analysis. This integrated lipidomic and proteomic study has allowed an in-depth analysis of the salt-induced regulation of membrane lipid metabolism.

Results

Separation of TP and PM by FFE

FFE was employed on microsomal membranes extracted from control and salt-treated ice plant (M. crystallinum L.) leaf tissue to isolate pools of distinct subcellular membranes for downstream lipidomic analysis (Figure 1A). FFE resulted in a high-resolution separation of microsomal membranes into 96 distinct fractions for each biological sample (6 in total: 3 biological replicates representing 3 individual pools of plants × 2 treatments). Every second fraction collected was then combined to give a total of 48 samples. Combined samples S08–S35 (original fractions 15–70) with positive protein values at an optical density of 280 (OD_{280}), and substantial endomembrane protein content (Guo et al., 2021) were used to confirm the origin of the membranes by quantitative proteomic analysis (SWATH-MS) of membrane marker proteins (Figure 1B).

Functional annotation of the identified M. crystallinum leaf membrane proteome was undertaken using the best BLASTP hits to sequences found in the National Center for Biotechnology Information (NCBI), using the UniprotKB/
SwissProt Arabidopsis (Arabidopsis thaliana) database (May 2020) as described in “Materials and methods.” The matched A. thaliana genome accessions were then submitted to the Subcellular Localization Database For Arabidopsis Proteins 4 (SUBA4) (Hooper et al., 2017) for subcellular localization information. Average FFE abundance profiles of the SUBA4 high confidence marker (HCM) (Supplemental Table S1) for resident proteins from TP, chloroplast, mitochondria, endoplasmic reticulum, Golgi, and PM from control and salt-treated plants are presented in Figure 1, C and D. Samples 13 and 25 were selected to represent TP, and PM for lipidomic analysis, respectively. These samples were not only TP or PM HCM-enriched but also had the least overlap with HCM from other subcellular compartments. Additionally, a combined reference sample was prepared, which consisted of equal amounts of each FFE sample to get a combination (Cmb) sample of all cellular membranes. This was then used as a positive control to monitor the global membrane lipid composition of ice plant leaf tissue in this study.

Lipid profiling of TP, PM, and combined membrane sample

For comprehensive and quantitative characterization of the lipidome of membrane samples, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC–ESI–MS/MS) was used to analyze the lipids extracted from three biological replicates of samples Cmb, TP, and PM taken from control and salt-treated plants. The lipidomic analysis identified a total of 19 lipid classes, including 11 phospholipids (PLs): phosphatidic acid (PA), phosphatidylycholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), and lysophosphatidylinositol (LPI); three glycolipids (GLs): monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG); and sterol glucosides (SGs), ceramide (Cer), glucosylceramide (GlcCer); and two neutral glycerolipids (NLs): triacylglycerol (TG), and diacylglycerol (DG) (Supplemental Table S2).

Since the physical properties of a lipid molecule can be influenced by its fatty acyl groups, a comprehensive study of the lipid molecular species comprised within each lipid class was also carried out in this work. Due to the limitations of the lipidomic analysis method, the positional distribution of the fatty acid (FA) and its double bond was not able to be determined, and thereby the identified lipid molecular species were annotated as: sum of carbon atoms in the FA chain(s): sum of double bonds in the FA chain(s). This resulted in 254 lipid molecular species identified across the 19 lipid classes (Supplemental Table S2).

The contribution of lipid class to the total amount of quantified lipids was calculated for LPC, LPE, LPI, LPG, PC, PE, PG, PI, PS, TG, and DG, based on the available
LSD demonstrated significant membrane-specific differences between the lipid profiles of the analyzed membrane samples (Figure 2A). As expected, PC and PE contributed the most to the total lipid in all three membrane types (together accounting for 65%–80%) (Figure 2A). However, the percentage of PC is remarkably higher in Cmb (57.4%) than in either the TP (33.4%) or the PM (25.9%), whereas the percentage of PE was the highest in PM (54.0%). PS was also the highest for PM (5.9%), which was over two and four times higher than that in TP (2.9%) and Cmb (1.3%), respectively. Not surprisingly, the proportion of PG in TP and PM (~3.0%) was dramatically lower compared with Cmb (15.0%), as PG is generally enriched in chloroplast membranes (Boudiere et al., 2014). PI content was relatively lower in all membrane types, despite its percentage being greater in Cmb (0.15%) and TP (0.13%), in comparison to PM (0.05%). For the quantified LPLs, the proportion of LPC was the lowest in TP (0.7%). Differences in LPE and LPG were only found between TP (1.2% and 0.7%) and PM (2.1% and 0.12%), whereas the percentage of LPI was the same in these two membrane samples (0.53% and 0.42%) and was significantly lower than Cmb (1.08%). The proportion of neutral lipids TG and DG were greater in TP, whereas there was no difference between Cmb and PM.

To investigate the contributions of all identified lipid classes to the variation of membrane types, the percent peak area relative to the total amount of lipid in each sample served as the basis for principal component analysis (PCA). Projections of the sample scores for the first and second principal components together account for 81.9% of the total variance (Figure 2D, scree plot in Supplemental Figure S1A). A clear separation was observed for nonoverlapping clusters corresponding to the different membrane types represented by their 95% confidence ellipses from three biological replicates. Biplot visualization indicates that TP showed a higher content of DG, LPA, and LPG, while greater proportions of LPC, LPE, PA, PS, PE, and Cer are the determining factors for PM. The lipids classes SQDG, MGDG, DGDG, PG, and LPI were positively correlated with Cmb (Figure 2D).

Salinity-induced lipidomic changes in TP, PM, and combined membrane sample
To elucidate the effect of salt treatment on membrane lipid profiles, the % peak area of identified lipid classes from three biological replicates of Cmb, TP, and PM isolated from control and salt-treated ice plant leaf tissue was submitted for PCA. There is a clear separation of the samples into two distinct clusters, in agreement with the treatment conditions for Cmb and TP (Figure 3, A and B), with PM showing a slight overlap between treatment ellipses (Figure 3C). The first two principal components explained 83.4%, 67.2%, 69.9% of the total variances of

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**Figure 2** Membrane-specific lipid profiles in the combined endomembrane (Cmb), TP and PM samples from leaf tissue of ice plant grown under control conditions. Bar charts represent the means ± standard deviations of three biological replicates of each A, PLs (lysophospholipid excluded), B, LPL, and C, NLs relative to total lipid content based on available standards. The means were compared using LSDs test with a P < 0.05, significant differences are indicated by different letters for each lipid class. D, PCA-biplot of all identified lipid classes based on their percent peak area. Sample scores for the first and second principal components were plotted, clusters corresponding to the different membrane classes were represented by 95% confidence ellipses from three biological replicates. The enlarged dots represent the means of the groups. The loading of each lipid class of the biplot was represented by arrows. The length of the arrow approximated the variance of each lipid class, and the angle approximated their correlations.
Salt treatment in Cmb, TP, and PM, respectively (scatter plot in Supplemental Figure S1, B–D).

Biplot visualizations demonstrated that a higher percentage of LPE, PE, and PS strongly contributed to the discrimination between control and salt treatment in Cmb (Figure 3A); a greater percentage of PC is the salient feature for TP under salt conditions; however, the proportions of most of the LPLs, GLs, PA, DG, SG, and GlcCer were negatively associated with salt treatment (Figure 3B). Further, salt treatment increased the percentage of PE, PS, PA, Cer, and GlcCer in PM, whereas there was a marked drop in the LPI pool (Figure 3C). However, although PCA results showed significant separations in accordance with treatments, hierarchical clustering (Ward, 1963) heatmap of percent peak area of identified lipid classes in Cmb, TP, and PM from three biological replicates of salt-treated plants compared to control. Red/blue: significantly (r test P < 0.05) increased/decreased; gray: unchanged. F, percent peak area of total PLs and NLs in three membrane types. Bar charts represent the means ± standard deviations of percent peak area of lipids from three biological replicates.

**Figure 3** Salt-induced lipidomic changes in the combined endomembrane (Cmb), TP and PM samples from leaf tissue of ice plant. Score plots of PCA model based on the percent peak area of identified lipid classes for membrane samples A, Cmb, B, TP, and C, PM, under control and salt treatment. Sample scores for the first and the second principal components were plotted, clusters corresponding to different treatments were represented by 95% confidence ellipses from three biological replicates. The enlarged dots represent the means of the groups. D, Hierarchical clustering (Ward, 1963) heatmap of identified lipid classes of Cmb, TP, and PM from three biological replicates of control and salt-treated plants. E, Log₂ FC of percent peak area of identified lipid classes in Cmb, TP, and PM from three biological replicates of salt-treated plants compared to control. Red/blue: significantly (t test P < 0.05) increased/decreased; gray: unchanged. F, percent peak area of total PLs and NLs in three membrane types. Bar charts represent the means ± standard deviations of percent peak area of lipids from three biological replicates.
of a particular lipid, ice plant also ensured the unique biological characteristics of individual subcellular membranes to maintain normal physiological activities upon salt treatment.

Specific changes to individual lipid classes are visualized by log, fold change (FC) (salt/control) represented as bar charts in Figure 3E. In agreement with the PCA-biplot results, the percentage of PE and PS were dramatically increased under salinity in Cmb (2.2- and 1.8-fold) and PM (1.3 and 1.3-fold), while increased PC was found in TP (1.2-fold). This resulted in an increase in total PL in TP, whereas the changes of percent peak area of NL in TP, and PL and NL in Cmb and PM were not significant (Figure 3F). This study detected a marked decrease in LPE in TP from salt-treated plants (0.8-fold), while this lipid increased in Cmb (1.8-fold). Furthermore, the percentage of GlcCer was significantly higher in PM from salt-treated plants.

A detailed survey of all lipid molecular species from each membrane sample was also undertaken in this study (Figure 4; Supplemental Figure S2). A total of 23 lipid molecular species were identified as significantly changed by salt in Cmb. Among which LPE 18:1, LPE 18:2, PE 34:2, PS 36:2, and PS 38:2 contributed to the observed increase in Cmb (1.8-fold). Among which LPE 18:1, LPE 18:2, PE 34:2, PS 24:0, MGDG 44:3, were measured in samples from Cmb from salt-stressed leaves (Figure 4). The lipid profile of Cmb also revealed changes to several molecular species for Cer, GlcCer, and neutral lipids: Cer 18:0/26:0, GlcCer 18:1/22:1, GlcCer 18:2/22:0, GlcCer 18:2/24:0, GlcCer 18:2/26:0, DG 36:4, and TG 50:1, all showed salt-induced increases in their percent peak area, whereas TG 53:2 was significantly reduced (Figure 4; Supplemental Figure S2).

In agreement with the percentage changes of total PS (Figure 3E), analysis of PM captured general increases in molecular species for PS, with five out of six species showing increases in log2FC value (FC of salt/control > 1) (Figure 4) under salt conditions; among which the alterations of PS 36:1 and PS 38:2 were significant (Figure 4). Similarly, 15 out of 17 molecular species for PE showed FC > 1 (Figure 4), supporting the higher proportion of total PE in PM from salt-treated plants (Figure 3E), despite that the changes of these molecular species were not statistically significant. The amount of three molecular species for PC, including PC 35:2, PC 35:4, and PC 36:2, decreased significantly in PM (Figure 4), even though the change of total PC was not significant (Figure 3E). Together with increased LPA 18:3, MGDG 34:3, TG 48:1, and several species for Cer and GlcCer, including Cer 18:0/22:1, GlcCer 18:1/18:0, GlcCer 18:2/18:0, GlcCer 18:2/24:1 (Figure 4; Supplemental Figure S2), the number of significantly altered molecular species totaled 16 in PM under salt stress conditions. Compared to sample Cmb and PM, the effect of salt treatment on TP lipids appears to be focused on specific lipid classes (Figure 4). Although only six molecular species showed significant alterations, 3 of them are molecular species of PC, including increased PC 34:2, PC 34:3, and PC 36:1, while the other three molecular species are decreased MGDG 36:4, MGDG 44:2, and increased 18:2 stigmasteryl glucoside (Figure 4; Supplemental Figure S2).

In plants, approximately 400 different fatty acyl species have been reported, with the composition varying between plant species but also within different plant tissues and even cell membranes (Sabudak et al., 2009; Li et al., 2017; Reszczynska and Hanaka, 2020). However, in general, 16:0 and 18:2 fatty acyl chains account for the highest percentage of the overall lipid content in plant membranes, followed by 18:3, 18:1, 18:0, and 16:1, whereas fatty acyl chains 14:0, 17:0, and 20:X are relatively rare (Guo et al., 2019). Our results suggested a possible increase in fatty acyl groups including 16:0, 18:1, 18:2, and 20:0 in Cmb from salt-treated plants, as the increased molecular species, such as PS 36:2, PS 38:2, DGDG 32:0, and TG 50:1 are most likely comprised by these FAs. This is supported by the increases of LPE 18:1 and LPE 18:2 (Figure 4). A higher percentage of these FAs was also observed in TP (PC 34:2 and PC 36:1) and PM (PS 38:2 and TG 48:1). In contrast, the proportion of molecular species containing four double bonds are likely to be reduced by NaCl not only in Cmb (PC 35:4 and PC 38:4) but also in TP (MGDG 36:4) and PM (PC 35:4), with only one exception—MGDG 34:4 increased in Cmb under salt stress (Figure 4).

Quantitative proteomics reveals salinity-induced regulations in ice plant leaf membrane proteome

SWATH-MS of the membrane proteomes from control and salt-treated ice plant resulted in the quantitative export of 1,493 unique proteins across all FFE samples, with 36 exclusively present in control plants, 31 exclusively present in salt plants, and 1,426 shared by both treatment conditions (Figure 5, A and B). The summed abundance of each protein across all FFE samples was used for statistical analysis to elucidate the global effects of salt treatment on ice plant leaf membrane proteome as described in “Materials and methods.” Proteins with an FC (salt/control) of >1.5 or <0.67 (1/1.5) and P < 0.05 were considered as differentially abundant proteins (DAPs). Accordingly, a total of 397 proteins showed significant protein abundance changes between salt-treated and control plants, with 87 increased and 310 decreased (Figure 5B; Supplemental Table S1), exclusively present proteins included). To investigate the function of the DAPs, significantly changed proteins were submitted to DAVID Bioinformatics Resources version 6.8 (Huang et al., 2009) for gene ontology (GO) pathway analysis.

Significantly enriched biological process (BP) GO terms totaled 24 for decreased DAPs (Figure 5C), including those relating to chloroplast and thylakoid membrane organization; metabolic processes, such as reductive pentose phosphate cycle, chlorophyll biosynthetic process, starch biosynthetic
process, lipid metabolic process, and RNA secondary structure unwinding; protein processes, including translation, translational initiation, regulation of translational initiation, formation of translation preinitiation complex, and ribosome biogenesis. Additionally, response to stress/stimulus, with response to cadmium ion and response to cold being the most significantly enriched terms. For proteins that increased in abundance, a total of 12 significantly enriched BP GO terms were identified. The most significantly enriched BP GO term is the abscisic acid-activated signaling pathway. GO terms associated with stress/stimulus responses, including cellular response to oxidative stress, response to reactive oxygen species, response to high light intensity, response to salt stress, response to cytokinin, and response to cadmium ion were significantly enriched. In addition, proteins with increased abundance were also enriched for GO terms, including photosynthesis, photoinhibition, oxidation–reduction process, and proton transport.

Salinity-induced regulations of lipid metabolism-related proteins

A total of 85 proteins (5.7% of identified proteins) were assigned as lipid metabolism-related, based on the Arabidopsis Acyl-Lipid Metabolism Database (Li-Beisson et al., 2013), and UniprotKB/SwissProt annotations (Supplemental Table S3). These proteins include those

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**Supplemental Figure S2:** Regulations of molecular species identified for other lipid classes.
involved in FA metabolism, glycerolipid synthesis, PL signaling, sterol synthesis, and oxylipin metabolism. Of these, 31 lipid metabolism-related proteins were shown to be responsive to NaCl treatment (FC ≥ 1.5 or FC ≤ 0.67, t test, \( P < 0.05 \)), among which 12 were significantly increased, while 19 were significantly decreased (Table 1).

Alterations to lipid metabolism-related proteins suggested that FA metabolism was responsive to salt treatment. For example, biotin carboxyl carrier protein of acetyl-coenzyme A (CoA) carboxylase 2 and Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, which are involved in de novo FA synthesis (Table 1), both were decreased in abundance in the salt-treated sample compared to control. Quantitative proteomics also observed a possible suppression in the FA desaturation pathway, as omega-6 FA desaturase (FAD6) was significantly reduced by salt treatment and temperature-sensitive sn-2 acyl-lipid omega-3 desaturase (FAD8) was identified only in plants under control conditions, whereas it was not detected in ice plant leaf membranes upon salt treatment (Table 1). Changes in...
## Table 1 Significantly regulated proteins involved in lipid metabolisms in membrane proteome of ice plant leaves by salt treatment

| Identifier | Uniprot Recommended Name | AGId | Log2FCd | P-Value | Regulation | Membrane Regulation Process | Subcellular Locationf |
|------------|---------------------------|------|---------|---------|------------|-----------------------------|-----------------------|
| iceplant_tr_239864 | Biotin carboxyl carrier protein of acetyl-CoA carboxylase 2 | AT5G15530 | –2.06 | 3.0E-02 | Decreased | FA synthesis | Plastid |
| iceplant_tr_16592 | Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha | AT2G38040 | –1.21 | 8.5E-03 | Decreased | FA synthesis | Plastid |
| iceplant_tr_246214 | Omega-6 FA desaturase | AT4G30950 | –1.54 | 9.3E-05 | Decreased | Prokaryotic galactolipid, sulfolipid, and PL synthesis | plastid |
| iceplant_tr_12325 | Temperature-sensitive sn-2 acyl-lipid omega-3 desaturase | AT5G05580 | –1.44 | 2.0E-03 | Decreased | Prokaryotic galactolipid, sulfolipid, and PL synthesis | Plastid |
| iceplant_tr_129303 | ABC transporter G family member 11 | AT1G17840 | INF | 1.6E-03 | Decreased | FA elongation and wax biosynthesis | PM |
| iceplant_tr_32681 | Nonspecific lipid transfer protein GPI-anchored 2 | AT3G43720 | –0.92 | 2.5E-02 | Decreased | FA elongation and wax biosynthesis | PM |
| iceplant_tr_17775 | Long chain acyl-CoA synthetase 1 | AT2G47240 | 1.01 | 4.2E-02 | Increased | FA elongation and wax biosynthesis | Cytosol |
| iceplant_tr_248764 | Long chain acyl-CoA synthetase 4 | AT4G23850 | –1.00 | 2.3E-03 | Decreased | Eukaryotic PL synthesis and editing | PM |
| iceplant_tr_125625 | Long chain acyl-CoA synthetase 4 | AT4G23850 | INF | 1.2E-04 | Decreased | Eukaryotic PL synthesis and editing | PM |
| iceplant_tr_246402 | Tocopherol cyclase, chloroplastic | AT4G32770 | 1.19 | 6.9E-03 | Increased | FA metabolism | Plastid |
| iceplant_tr_16993 | Glycerophosphodiester phosphodiesterase GDPDL3 | AT4G26690 | –1.18 | 4.9E-02 | Decreased | Lipid metabolism | PM |
| iceplant_tr_127069 | LPP2 | AT1G15080 | 1.40 | 3.1E-02 | Increased | Prokaryotic galactolipid, sulfolipid, and PL synthesis | PM |
| iceplant_tr_18579 | Glycerol-3-phosphate acyltransferase | AT1G32200 | –0.45 | 9.1E-03 | Decreased | Prokaryotic galactolipid, sulfolipid, and PL synthesis | Plastid |
| iceplant_tr_133123 | Non-specific phospholipase C4 | AT3G03530 | –0.94 | 3.0E-02 | Decreased | Eukaryotic galactolipid and sulfolipid synthesis | PM |
| iceplant_tr_96650 | Protein trigalactosyldiacylglycerol 1 | AT1G19800 | INF | 1.4E-04 | Increased | Prokaryotic galactolipid, sulfolipid, and PL synthesis | Plastid |

(continued)
Table 1 Continued

| Identifier       | Uniprot Recommended Nameb | AGIc | Log,FCd | P-Value | Regulation                        | Membrane Regulation Process | Subcellular Locationf |
|------------------|----------------------------|------|---------|---------|-----------------------------------|-----------------------------|-----------------------|
| iceplant_tr_10766 | Protein trigalactosydialglycerol 2 | AT3G20320 | -0.62   | 1.2E-02 | Decreased                        | and sulfolipid synthesis    | plastid               |
| iceplant_tr_80212 | Ethanolamine-phosphate cytidylyltransferase | AT2G38670 | 2.30    | 2.9E-02 | Increased                        | Lipid trafficking           | mitochondrial         |
| iceplant_tr_125688 | Inositol-3-phosphate synthase isozyme 2 | AT2G22240 | -1.10   | 2.5E-02 | Decreased                        | Eukaryotic PL synthesis and editing | unknown               |
| iceplant_tr_242403 | 1-acyl-sn-glycerol-3-phosphate acyltransferase 2 | AT3G57650 | 1.61    | 2.4E-03 | Increased                        | Eukaryotic PL synthesis and editing | ER                    |
| iceplant_tr_76295 | Phospholipid:diacylglycerol acyltransferase 1 | AT5G13640 | 0.47    | 3.6E-02 | Increased                        | TG biosynthesis             | ER                    |
| iceplant_tr_11379 | Wax ester synthase/diacylglycerol acyltransferase 1 | AT5G37300 | 0.80    | 2.3E-02 | Increased                        | FA elongation and wax biosynthesis | Peroxisome,cytosol |
| iceplant_tr_81209 | Caffeoylshikimate esterase | AT1G52760 | -0.88   | 2.3E-02 | Decreased                        | TG and FA degradation       | Mitochondrion         |
| iceplant_tr_19023 | Phospholipase A 1-delta | AT2G42690 | -0.61   | 3.0E-02 | Decreased                        | Oxylipin metabolism         | Cytosol               |
| iceplant_tr_254666 | Phosphonoiotide phospholipid C 4 | AT5G58700 | -1.62   | 1.1E-02 | Decreased                        | PL signaling                | PM                    |
| iceplant_tr_24046 | Phospholipase D alpha 1 | AT3G15730 | -0.69   | 7.7E-03 | Decreased                        | PL signaling                | Cytosol               |
| iceplant_tr_19120 | Cycloartenol-C-24-methyltransferase | AT5G13710 | 1.15    | 1.9E-03 | Increased                        | Sterol synthesis            | Golgi                 |
| iceplant_tr_128900 | Cycloartenol-C-24-methyltransferase | AT1G11100 | 2.42    | 2.5E-02 | Increased                        | Sterol synthesis            | ER                    |
| iceplant_tr_102394 | Ankyrin repeat domain-containing protein 28 | AT2G17390 | -0.71   | 3.7E-02 | Decreased                        | Lipid binding               | Nucleus               |
| iceplant_tr_264677 | Patellin-4 | AT1G30690 | 0.63    | 1.2E-02 | Increased                        | Lipid binding               | Cytosol               |
| iceplant_tr_247929 | SYT4 | AT2G11100 | 1.81    | 2.5E-02 | Increased                        | Lipid binding               | ER                    |
| iceplant_tr_126434 | Pyridoxal 5'-phosphate synthase subunit PDX1.3 | AT5G01410 | -0.91   | 4.2E-03 | Decreased                        | Lipid protection            | Cytosol               |

aTable showing only the significantly regulated proteins involved in lipid metabolisms in the membrane proteome of ice plant leaves by salt treatment. Refer to Supplemental Table S3 for the information on all lipid metabolism-related proteins.
bSequences of M. crystallinum proteins were submitted to NCBI BLAST and compared against the UniprotKB/Swissprot database; descriptions are based on UniprotKB/Swissprot A. thaliana annotations.
cAGIs are the Arabidopsis Genome Initiative codes for the matched Arabidopsis genes.
dFold change of proteins that were exclusively present in control/salt-treated plants is shown as INF corresponding to decreased/increased in the “Regulation” column.

abundance also occurred in several proteins that participated in FA elongation and wax biosynthesis. For instance, there was a marked decrease in ABC transporter G family member 11, nonspecific lipid transfer protein GPI-anchored 2, and long-chain acyl-CoA synthetase 4 in salt-treated plants. However, the abundance of long-chain acyl-CoA synthetase 1 was elevated in plants treated with NaCl (Table 1).

Salt treatment also induced changes in glycerolipid synthesis in the ice plant leaf membrane proteome, which included alterations in proteins pertaining to glycerophospholipid, glycolglycerolipid, and TG lipid synthesis pathways. Salt-induced proteins that are involved in these pathways include lipid phosphate phosphatase 2 (LPP2), protein trigalactosydialglycerol 1, ethanolamine-phosphate cytidylyltransferase (PECT), 1-acyl-sn-glycerol-3-phosphate (G3P) acyltransferase 2, phospholipid:diacylglycerol acyltransferase 1, and wax ester synthase/diacylglycerol acyltransferase 1. While proteins such as glycerophosphodiester phosphodiesterase GDPDL3, G3P acyltransferase, nonspecific phospholipase C4, protein trigalactosydialglycerol 2, and caffeoylshikimate esterase showed reduced abundance upon salt treatment (Table 1). Moreover, the comparison between proteomes reveals that the sterol synthesis pathway was induced by salt treatment in iceplant leaf membrane extracts, as the abundance level of cycloartenol-C-24-methyltransferase and cytochrome P450 51G1 significantly increased.

Discussion

Membrane-specific differences in lipid composition of different membrane types

PL composition of the combined sample of all FFE fractions (Cmb) from leaves of ice plant in this study showed a similar lipid profile to that previously reported from a cell-type-specific total lipid extraction of epidermal bladder cells (EBCs) of this plant (Barkla et al., 2018) and rosettes of Arabidopsis (Have et al., 2019). PC accounted for the highest percentage of lipids, followed by PE, PG, and PS, with LPLs comprising minor components. However, the exception to
this was the percentage of PI, which was much lower in the leaf tissue compared to the EBC. In plants, inositol containing PLs, especially the polyphosphoinositides, play an important role in nuclear regulation and cell signaling (Munnik and Vermeer, 2010; Dieck et al., 2012; Gillaspy, 2013; Jia et al., 2019). These results indicate that compared to the whole leaf membrane extraction, EBC may have a more complex signaling response, whereby PIs are required for the activation of EBC-specific stress tolerance pathways (Aharonovitz et al., 2000).

Lipid profiles of TP, PM, and Cmb from ice plant leaf tissue under control conditions demonstrated membranespecific differences in their composition (Figure 2). One of the most striking features of TP is that it is comprised of dramatically lower total PL but higher total NL compared with PM and Cmb (Figure 6, A and B), which was mainly due to the greater proportion of TG in TP (Figure 2C). TG has been shown to be an integral component of cell membranes and is the predominant energy storage molecule in plants (Lerique et al., 1994). This may indicate that TP is likely to serve as a lipid reservoir, similar to plant plastoglobules, supplying lipid building blocks for membrane expansion by releasing TG (Rottet et al., 2015). Recent studies have discovered additional roles for TG in plant vegetative tissue, including the involvement in lipid breakdown, FA turnover, and stress response (Fan et al., 2017; Barkla et al., 2018). Indeed, the hydrolysis of TG to free FA to integrate into PLs to form new membranes is likely to be required by TP to accommodate vacuole expansion as a function of salt-accumulation in leaf cells of this halophyte under salt treatment (Adams et al., 1992; Bremberger and Lüttinge, 1992).

Notably, a greater PC to PE ratio in Cmb was a result of a higher proportion of PC in this sample (Figures 2, A and C). Structurally, membrane lipids can be classified into cylindrical shape bilayer forming lipids (e.g. PC and DGDG) and conical shape nonbilayer forming lipids (e.g. PE and MGDG). Bilayer lipids ensure membrane stability, while nonbilayer lipids are essential for biochemical processes (Frolov et al., 2011; Zick et al., 2014). A balanced bilayer and nonbilayer lipid ratio is a key feature for all biological membranes (Larsson et al., 2006; Perlikowski et al., 2016). As TP and PM have much lower PC/PE ratios compared to that of the Cmb sample (which also contains TP and PM along with all other subcellular membranes), it suggests that the presence of a higher proportion of nonbilayer lipids would be important for the functional activity of proteins and lends the membrane high plasticity (Brown, 2012; Dlouhy et al., 2020).

Unlike the other subcellular membranes, which are abundant in PC and PE, PG is the predominant PL in photosynthetic membranes (Joyard et al., 1998). This is confirmed in our study as the lipid content of PG in Cmb, which contained photosynthetic membranes, was over five times higher than that in TP and PM (Figure 2A), and its percent peak area was also the highest in Cmb (Figure 6D). Previous studies have shown that PG is a vital component of photosynthetic membranes since the disruption to PG biosynthesis was accompanied by the defective development of chloroplasts and impaired photosynthesis (Kopecna et al., 2015; Kobayashi et al., 2016; Lin et al., 2016). A few studies have also demonstrated that PG is needed for the activation of the enzyme MGDG synthase that is involved in the biosynthesis of GLs (Dubots et al., 2010; Nitenberg et al., 2020).
including MGDG, DGDG, and SQDG, which are the main lipid constituents of photosynthetic membranes (Kalisch et al., 2016). The PCA-boxplot suggested that PG and GLs in this study were positively correlated, as indicated by their arrow angles in Figure 2D. Accordingly, MGDG, DGDG, and SQDG had the highest percent peak area in the Cmb relative to TP and PM (Figure 6, E–G). This also suggested that, in addition to PG, and as expected, GLs also comprise a significantly greater proportion in the Cmb compared to TP and PM (Figure 6H).

Salinity effects on lipid profiles of different membrane types

High salinity has been shown to alter the content of total PL in membranes, with results showing both increases and decreases, possibly due to the different plant species/tissues, membrane types, and NaCl concentration, as well as lipid characterization methods employed in these studies (Mansour et al., 1994; Lin and Wu, 1996; Elkahoui et al., 2004; Salama and Mansour, 2015; Barkla et al., 2018). Our data show that no significant alteration to the percent peak area of total PL was observed in PM and Cmb (Figure 3F), whereas that of TP was elevated by the salt treatment, which is mainly caused by the increased percentage of PC in this membrane (Figure 3E).

In plant cells, both PC and PE are the predominant components of all membranes with the exception of photosynthetic membranes. Studies have confirmed the positive role of membrane PC in plant salt tolerance; for example, significantly increased amounts of PC were observed in EBC of salt-treated ice plant (Barkla et al., 2018) and PM from salt-cocultured PM, regulating membrane protein localization, and influencing the binding and activity of peripheral membrane proteins (Yeung et al., 2008; Platre et al., 2018; Casares et al., 2019). Importantly, the anionic properties of PS have been shown to confer the membrane a higher binding affinity for the stress signaling molecule Ca\(^{2+}\) (Lin and Wu, 1996), which in salt-stressed plants plays a key role in activating the salt-overly sensitive (SOS) pathway (Zhu, 2002).

Interestingly, PS is also capable of adopting a hexagonal phase (nonbilayer configuration) under conditions of reduced pH or elevated Ca\(^{2+}\) concentration (Hafiez and Cullis, 2001; Dowhan and Bogdanov, 2002; Fuller et al., 2003). This may explain why in contrast from that in the PM and Cmb, the PE proportion remained unchanged in TP (Figure 3E), as the higher nonbilayer proportion required by membrane salt tolerance in TP could be supplemented by PS of nonbilayer status since the vacuole generally possesses an acidic pH and high Ca\(^{2+}\) concentration (Schonknecht, 2013; Shen et al., 2013).

Sterols are essential components of plant membranes, although their physical and biological role in membrane remodeling is not very well characterized. Previous studies have shown that membranes isolated from salt-tolerant species are rich in sterols, whereas species that are considered to be salt-sensitive have low amounts of sterols and increases in sterol content as a result of NaCl treatment have been reported for membranes of salt-tolerant species, while sterol content of salt-sensitive species generally decreases with salt treatment (Blits and Gallagher, 1990; Kerkeb et al., 2001; Guo et al., 2019; Sarabia et al., 2020). In support of these findings, mutations to genes involved in sterol biosynthesis reduced the salt tolerance of yeast,
resulting in PM hyperpolarization and cytosolic acidification (Kodedova and Sychrova, 2015). In this study, increased amounts of most SG molecular species, reflected by positive Log₂FC values, were observed in Cmb and PM under salt stress, although they were not found to be statistically significant (Supplemental Figure S2). The role of sterols in TP membranes is largely unknown, and in this study, there was little change in either total SG or SG molecular species under salt treatment, suggesting that they do not play a role in the lipid response of this membrane (Figure 3E; Supplemental Figure S2).

The change in fatty acyl profiles of membrane lipids has also been shown to be crucial for plant salt tolerance (Ben Hamed et al., 2005; Sui and Han, 2014). Lipidomic analysis captured a higher percent peak area of fatty acyl species 16:0, 18:0, 18:1, and 18:2 in TP, PM, and Cmb samples from salt-treated plants (Figure 4; Supplemental Figure S2). These are likely precursors for 32:0, 34:2, 36:1, 36:2, 38:2, and 50:1 species. This may indicate a salt-stimulated de novo synthesis of long-chain FAs (Li-Beisson et al., 2013) or decreased desaturation process for polyunsaturated fatty acyl species in membranes from salt-treated plants, as was shown by a proportionate reduction in polyunsaturated molecular species, such as lipid that contains FAs 35:4, 36:4, and 38:4 (Figure 4; Supplemental Figure S2). But either way, regulated saturation of membrane lipids could possibly change the liquid order status and thereby the fluidity of membranes (Harayama and Riezman, 2018). While salt-induced regulation of membrane lipid saturation has been reported in a number of studies, it is still not easy to draw a conclusion on the regulation of membrane lipid saturation level, as the changes were inconsistent between the different species or tissues studied (Sun et al., 2010; Sui and Han, 2014; Omoto et al., 2016). Due to technical limitations of the lipidomic analysis method and the lack of commercial standards, the absolute quantification of the lipids and thereby the regulation of saturation level of membrane lipids by salt stress was not possible in this study.

Integrated lipidomic and proteomic analyses reveal membrane remodeling in ice plant under salinity

FA chains are essential parts of all acyl lipids, and evidence has shown that changes to the properties of fatty acyl chains influence lipid regulation and plant salt tolerance (Zhang et al., 2012; Tsydendambaaev et al., 2013; Sui and Han, 2014; Omoto et al., 2016). Unlike in other eukaryotes, de novo FA synthesis in plants occurs in plastids rather than in the cytosol (Li-Beisson et al., 2013); thereby, efficient FA synthesis largely relies on proper chloroplast function (Heredia-Martinez et al., 2018). Quantitative proteomic data comparing salt-treated to untreated samples demonstrated that the proteins with decreased abundance were significantly enriched in GO terms for chlorophyll biosynthetic processes, chloroplast organization, and more specifically, thylakoid membrane organization processes (Figure 5C). This suggested there may be inhibition of chloroplast processes, including photosynthesis in leaves from salt-treated ice plant (Barker et al., 2004; Cushman et al., 2008). Not surprisingly, results in this study suggested a suppressed FA synthesis, as a dramatic decrease was observed in biotin carboxyl carrier protein of acetyl-CoA carboxylase 2 (BCCP2) and acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (CAC3; Table 1; Figure 7A), both components of the acetyl-coenzyme A carboxylase complex involved in the initial step of FA synthesis (Elborough et al., 1996).

Moreover, two plastid localized FA desaturases, FAD6 and FAD8, were also reduced in abundance by salt treatment in ice plant leaf tissue (Table 1; Figure 7A). FAD6 converts oleic acid (18:1) to linoleic acid (18:2) by inserting a double bond at the ω-6 position, while FAD8 converts linoleic acid (18:2) to linolenic acid (18:3) by inserting a double bond at the ω-3 position. Correspondingly, lipidomic data demonstrated a decreased percentage of polyunsaturated lipid molecular species in the plant under salt treatment (Figure 4). This could be the reason for the increased proportions of fatty acyl species like 16:0, 18:0, and 18:1 (Figure 4), even though there may be an overall suppressed FA synthesis in the salt-treated samples. Interestingly, FAD genes, including FAD2, FAD3, FAD6, and FAD7, have been shown to be required for plant salt tolerance, although these studies were mainly carried out in salt-sensitive plants (Im et al., 2002; Zhang et al., 2005, 2009, 2012; Wang et al., 2014), indicating FADs may be differently regulated in glycophytes versus halophytes. As an example, in a comparative study of two barley (Hordeum vulgare) varieties differing in their salt tolerance, it is proposed that rather than increase the unsaturation degree of the membrane, the ability to maintain membrane integrity is more important for plant salt tolerance since the elevated unsaturation to saturation ratio of membrane lipids in the salt-sensitive variety was not observed in the more salt-tolerant variety (Chalbi et al., 2013).

In agreement with lipidomic results, which showed salt-induced alterations to glycerolipids, quantitative proteomics by SWATH-MS analysis confirmed a highly salt-responsive glycerolipid metabolism in leaves. Pathways of de novo synthesis of PC and PE, collectively known as the Kennedy pathway (Kennedy, 1956), starting with the phosphorylation of choline/ethanolamine by choline/ethanolamine kinase, and ending with the conjugation of CDP-choline/ethanolamine and DG into PC/PE by choline/ethanolamine phosphotransferase, appeared to be induced by salt in this study (Figure 7B). Specifically, PECT1, which catalyzes the formation of CDP-ethanolamine from ethanolamine phosphate, was induced by salt treatment (Table 1). This agrees with the results, which showed increases in total PE and its individual molecular species in both the Cmb and PM fractions under salt stress (Figures 3, E and 4).

Salt-stimulated synthesis of PE was supported by the increased abundance of LPP2, which directly dephosphorylates PA to generate the precursor DG for PC and PE synthesis (Pierrugues et al., 2001; Figure 7B). This may also contribute
to an increased synthesis of TG since the abundance of the enzyme phospholipid:diacylglycerol acyltransferase 1 (PDAT1), which catalyzes the acyl-CoA-independent formation of TG from DG (Dahlqvist et al., 2000), was dramatically induced by salt treatment (Figure 7B). Interestingly, lipidomic data did not support an increased amount of total TG in the Cmb sample in the current study (Figure 3E). It is possible that the abundance of EBCs in the leaf microsomal extracts may be the cause of the overall unchanged total TG in the Cmb sample, as flow cytometry, lipidomic, and transcriptomic data together all showed a degradation rather than biosynthesis of TG in this single cell type from ice plant under salt conditions (Barkla et al., 2018).

Generally, as the precursor of PLs, PA is synthesized by two pathways: either through the incorporation of fatty acyl chains and G3P or the conversion from PC through the action of phospholipase D (PLDs). Proteomic results showed an increased abundance of lysophosphatidyl acyltransferase 2 (1-acyl-sn-G3P acyltransferase 2), which converts LPA into PA by incorporating an acyl moiety at the sn-2 position in plants (Kim et al., 2005a, 2005b), suggesting an increased synthesis of PA. However, in leaves, the results described by proteomic data show the pathway derived from PC seemed to be inhibited by salt treatment due to the dramatic decline in PLD alpha 1 (PLDα1; Figure 7B). This agrees with the observation of an elevated level of PC or PE in profiled membrane samples (Figure 3E). Furthermore, decreased
amounts of nonspecific phospholipase C4 from salt-treated plants also implied a suppressed metabolic flow from PC and PE to DG (Figure 7B).

In addition to its metabolic role, PLD has also been shown to take part in signaling events in plants in response to abiotic stress (Hong et al., 2010; Guo and Wang, 2012; Zhao, 2015; Liu et al., 2017). The most abundant member of the family of PLDs (Fan et al., 1999), PLDx1, was shown to be essential for stress tolerance, as Arabidopsis became more sensitive to salt treatment when this gene was mutated (Bargmann et al., 2009). PLDx1 mediates salt stress signaling through the regulation of mitogen-activated protein kinase, which phosphorylates the Na+/H+ exchanger, SOS1, in plants under salt stress (Yu et al., 2010; Vadovic et al., 2019). However, our data demonstrated that neither the percentage of lipid PA nor the abundance of the SOS1 protein was significantly regulated by salt treatment (Figure 3E; Supplemental Table S1). Thereby, suppressed PLDx1 protein abundance may be a strategy adopted by ice plant leaf tissue for maintaining adequate levels of the PLs PC and PE in membranes of leaf tissue under salt treatment (Figure 7B).

In contrast to the positive role of PLD in stress responses, the signaling molecule phosphoinositide phospholipase C4 (PLC4) is considered to play a negative role in osmotic stress response (Xia et al., 2017). The proteomic data supports this, as the abundance of PLC4 was reduced in the salt-treated membrane proteome (Table 1). A recent study demonstrated that the overexpression of PLC4 caused a decline in inositol PLs in rice (Oryza sativa) under salt stress conditions, suggesting PLC4 may be involved in the regulation of inositol PL signaling (Deng et al., 2019). Increased inositol PL signaling was also believed to be involved in the stress tolerance response in EBC of salt-treated ice plant (Barkla et al., 2018), where downregulation of the genes for both PLDx1 and PLC4 was captured by transcriptomic analysis.

While sterols are structural components that are important for membrane fluidity and permeability, intermediates in the sterol biosynthesis pathways can also act as signaling molecules in plants in response to abiotic stresses (Valitova et al., 2016; Rogowska and Szakiel, 2020). In agreement with the general increase in molecular species for sterols in the salt-treated samples reflected by positive Log2FC of all sterol molecular species (Supplemental Figure S2), induced sterol synthesis is also supported by significantly increased cycloartenol-C-24-methyltransferase (SMT1) and cytochrome P450 51G1 (CYP51G1; Figure 7C). These enzymes catalyze the initial step and the postqualene reaction of sterol biosynthesis, respectively (Diener et al., 2000; Kim et al., 2005a, 2005b). In rice, mutations in the CYP51 gene, encoding an obtusifoliol 14α methyl demethylase, resulted in greater salt sensitivity, measured by increased relative conductivity of electrolytes leaked from whole seedlings (Xia et al., 2015). It is possible to speculate that this increased salt sensitivity may be a result of interrupted stress signaling transduction pathways as studies have shown that the spraying of obtusifoliol on potato (Solanum chacoense) leaves significantly induced mRNA levels of CYP51G1 locally and systemically, suggesting this transient biosynthetic intermediate can act as a bioactive lipid signaling molecule (O’Brien et al., 2005).

The data from proteomic analysis suggested that proteins that take part in lipid transport are also affected by salt treatment (Figure 7D; Table 1). For example, the protein transporters involved in the export of suberin to the extracellular matrix, including nonspecific lipid transfer protein GPI-anchored 2 (LTPG2) and ABC transporter G family member 11 (ABCG11) (Luo et al., 2007; Panikashvili et al., 2010; Kim et al., 2012) were decreased in the salt-treated plants. In Arabidopsis, increased root suberisation was shown to lead to reduced uptake of inorganic nutrients such as Ca2+ (Baxter et al., 2009; Wang et al., 2020), suggesting conversely, the reduced transport of suberin in this study may lead to increases in Ca2+ uptake and signaling as a salt stress adaptation response for maintaining ionic and osmotic homeostasis. In agreement with this study, transcriptomic data from the ice plant EBC also demonstrated a higher percentage of downregulated transcripts involving FA elongation, wax biosynthesis, and suberin synthesis under salt treatment (Oh et al., 2015; Barkla et al., 2018).

Salt treatment also resulted in changes in abundance to lipid binding and trafficking proteins (Figure 7). In particular, synaptotagmin-4 (SYT4) was induced by salt treatment (Table 1). Studies have shown that SYTs play an important role in maintaining PM integrity for both calcium-dependent freezing and osmotic tolerance in Arabidopsis leaves (Schapire et al., 2008; Yamazaki et al., 2008). SYTs are proposed to protect membranes by triggering calcium-dependent membrane resealing of the damaged sites (Yamazaki et al., 2008). Furthermore, increased Patellin-4 (PATL4) in this study may indicate enhanced signal transduction in ice plant upon salt treatment, as it has been shown to accumulate at the cell plate and bind inositol PLs (Peterman et al., 2004). In addition, evidence has shown PATL as a membrane trafficking-related protein, negatively regulating PM Na+/H+ activity in Arabidopsis, where the T-DNA insertion mutant patl1 displayed a salt-sensitive phenotype and a disruption in cellular redox homeostasis (Zhou et al., 2018).

Ice Plant is a facultative crassulacean acid metabolism (CAM) plant, with the change in metabolism from C3 to CAM triggered by salt stress (Guan et al., 2020). As membrane transport is a key feature of CAM, it is not surprising that we identify changes in abundance of key CAM enzymes in our study. Of particular note is the TP V-ATPase, which provides the electro-chemical gradient driving secondary active transport at the vacuole membrane. In this study, we identified 12 subunits with two significantly increased in abundance with salt stress including VHA-A and VHA-D (Supplemental Table S1). Recent work on this enzyme in nonplant eukaryotes has demonstrated the importance of lipid/protein interactions and shown that the low-abundant phosphoinositide, PI 3,5-bisphosphate (PI(3,5)P2) has a dramatic effect on the assembly of the V-ATPase and its...
activation under salt stress (Li et al., 2014). Whether a similar interaction is involved in plants remains to be demonstrated.

Conclusions
The importance of the chemical diversity and abundance of membrane lipids in sustaining organelle structure and function has been well studied in humans due to the association of lipids with various diseases (Harayama and Riezman, 2018). However, progress in understanding the role of membrane lipids in plant abiotic stress tolerance has been minimal, especially in relation to salt tolerance. This study highlights the benefit of combining innovative FFE fractionation techniques with tandem mass spectrometry-based quantitative approaches for subcellular membrane lipidome and proteome profiling in leaves of halophyte *Mesembryanthemum crystallinum* in a high-throughput manner. Results allowed the characterization of salt-induced changes to subcellular membrane proteins and lipids to identify mechanisms important for responding to salinity. Linking the series of changes identified in this study to other metabolic processes, for instance, salt stress signaling, protein–lipid interactions, membrane communication, and alterations in membrane physical properties, to decipher the physiological responses occurring in the plants in a more comprehensive perspective will be the next level of challenge.

Materials and methods

Plant materials and growth conditions
Ice plant (*Mesembryanthemum crystallinum* L.) seeds were germinated in the potting substrate in a seedling propagation tray, as described in our recent study (Guo et al., 2021). Three weeks following germination, the seedlings of uniform size were transplanted to pots containing the same soil mixture at a density of two plants per 15-cm diameter pot. The watering regime consisted of daily tap water with a weekly supply of one-half Hoagland’s medium. For the salt treatment, 6-week-old plants were treated with NaCl (200 mM) daily for 2 weeks which allowed the plant to complete the morphological and physiological changes from C3 photosynthesis to CAM photosynthesis under salt stress (Guan et al., 2020). The glasshouse for growing plants was equipped with an evaporative cooling system to maintain the temperature between 15°C and 32°C and was placed under natural irradiation and photoperiod. The second pair of mature leaves was harvested once the plants were 8 weeks old.

FFE of microsomal membrane extracts
The experimental conditions of microsomal membrane extraction were described in detail in our recent study (Guo et al., 2021). Briefly, leaf material (60 g) from control and salt-treated ice plant was homogenized in an ice-cold homogenization buffer (Guo et al., 2021) using a commercial blender. Samples were filtered through cheesecloth and centrifuged at 10,000g for 20 min at 4°C. The pellets were discarded, and the supernatants were concentrated by centrifugation at 100,000g for 50 min at 4°C.

Microsomal membranes were diluted 2:1 (v:v) in a separation medium containing 250 mM sucrose, 10 mM acetic acid, 10 mM triethanolamine, and 2 mM KCl, and centrifuged at 14,000g for 20 min at 4°C prior to injection into FFE chamber. Diluted samples were injected continuously through a peristaltic pump at a rate of 1.2 mL h⁻¹ via the anodic sample inlet while media inlet composition was as follows: inlets 1 and 9, stabilization medium comprising 180 mM sucrose, 40 mM acetic acid, 40 mM triethanolamine, and 8 mM KCl; inlets 2–8, separation medium comprising 250 mM sucrose, 10 mM acetic acid, 10 mM triethanolamine, and 2 mM KCl: cathodic and anodic circuit electrolyte solutions consisted of 100 mM triethanolamine, 100 mM acetic acid, and 10 mM KCl adjusted to pH 7.4 with NaOH. In order to avoid the loss of chloride by anodic oxidation, formaldehyde (0.4% v/v) was added to the anodic solution. The counterflow medium was the same as the separation medium for inlets C1, C2, and C3. FFE was conducted horizontally at a constant voltage of 750 V (~135 mA) with a media and counterflow rate of 250 mL h⁻¹. The chamber temperature was maintained at 5°C by the continual flow of coolant from a circulating water bath.

Each injected sample was fractionated into 96 fractions which were then continually collected into 15 mL polypropylene tubes placed on ice. Every second fraction was then combined to give a total of 48 protein samples (i.e. fractions 1 and 2, 3 and 4, 5 and 6, etc.). Combined samples S08–S35 (original fractions 15–70) with positive protein values at OD380 and substantial endomembrane protein content (Guo et al., 2021) were used to confirm the origin of the membranes by identifying membrane marker proteins using quantitative proteomic approaches.

TCA precipitation and trypsin digestion of membrane proteins
Two hundred microliter 10× TE, 0.3% (w/v) sodium deoxycholate, and 72% (w/v) trichloroacetic acid were added to 1 mL of each sample in a centrifuge tube in sequence, and vortexed between every step. Samples were incubated on ice for 1 h, and the supernatant was discarded by aspiration after centrifugation in a Sigma 4K15 laboratory centrifuge at 14,000 rpm for 20 min at 4°C. Pellets were resuspended with 90% methanol then incubated overnight at −20°C. Following the repeated steps of centrifugation and aspiration, protein pellets were dried in a fume cabinet on ice. Samples were made up to 1 mg mL⁻¹ concentrations using 2 M urea in 50 mM ammonium bicarbonate (pH 8.0). One hundred microliters of each sample were digested with 20-μg trypsin and incubated in an ultrasonic bath for 10 min. Tryptic digestion was carried out overnight at 37°C followed by 4 min in a microwave on the lowest power setting. Samples were dried down using a Heto vacuum centrifuge at 45°C for 2 h or until dry and dissolved in 100 μL 1% trifluoroacetic acid in Milli-Q water, 50 μL samples were aliquoted into autosampler vials for injecting into the nano HPLC/MS MS/MS system.
LC–MS/MS analysis of membrane proteins

The tryptic peptide samples were analyzed using Ekspekt nano LC400 uHPLC (SCIEX, Canada) coupled with a TripleTOF 6600 quadrupole time-of-flight (QTOF) mass analyzer (SCIEX, Canada) equipped with a PicoView nanoflow (New Objective, USA) ion source. A trap column (5 mm × 300 μm, C18 3 μm, SGE, Australia) and an analytical column (75 μm × 150 mm ChromXP C18 CL 3 μm, SCIEX, Canada) were used. A linear gradient consisting of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) was employed with Solvent B from 2% to 40% over 60 min, 40%–90% for 5 min, 90% for 5 min and equilibration at 2% between samples. The mobile phase flow rate was 400 nl min⁻¹, and the column temperature was set at 45°C. The peptide samples (5 μL each) were loaded onto the trap column (10 μL min⁻¹, 5 min), and eluted onto the analytical column at 400 nL min⁻¹. The ionization parameters were as follows: interface heater 150°C, ionspray voltage 2,600 V, declustering potential 80 V, and both nebulizer gas 1 (GS1) and curtain gas flow values set at 30.

The protein identification for peptide library production was achieved using the information-dependent acquisition (IDA) mode of the QTOF. During the analysis, a full scan TOF (50 ms, 350–1,800 m/z) was first acquired, and then the 30 most intense ions (with minimum 200 counts and a charge state of +2 to +5) of the TOF scan were selected to generate MS/MS spectra (100 ms each, with a rolling collision energy, product ion scan 100–1,500 m/z) using the QTOF. An Analyst TF 1.7 software (SCIEX, Canada) was used for data acquisition and analysis.

Peptide and protein quantification data were acquired under the same HPLC and mass spectrometry experimental conditions as described for IDA, with the following exceptions. Data were acquired using a SWATH, product ion MS/MS all approach. The SWATH experiment was set to acquire 100 product ion spectra from m/z 350 to 1,500 per scan cycle with a product ion window set to 6 Da and collision energy from 16 to 60 V with an energy spread of 5 V. The TOF-MS scan acquisition time was set to 50 ms and each product ion scan to 25 ms. The data were also acquired and processed using Analyst TF version 1.7 software (SCIEX, Canada).

For spectral library generation, all mass spectrometry files (28 samples × 2 treatments × 3 biological replicates representing three individual pools of plants) were searched in unison in ProteinPilot software (Version 5.0.2, SCIEX) using the paragon algorithm. Samples were uploaded as unlabeled samples using the following parameters: protein identification, digestion with trypsin, and no special factors. The search was performed by searching an ice plant genomic database of all M. crystallinum ORFs obtained from Prof. John Cushman (University of Nevada). Peptides with confidence > 95% were selected, which generated a final spectral library containing 1,917 distinct proteins at a critical false discovery rate of 1%. Spectral alignment and targeted data extraction of SWATH-MS data were conducted in the SWATH Processing Micro App in PeakView (Version 1.2, SCIEX) using the spectral library generated above. Raw files were loaded using an extraction window of 15 min with the following parameters: six peptides, five transitions, and peptide confidence of > 95%, excluded shared peptides, and extracted ion chromatogram width set at 75 ppm.

Bioinformatics analysis of membrane proteome

Functional annotation of the identified ice plant proteome was based on the best BLASTP hits found in the NCBI, UniprotKB/SwissProt Arabidopsis database (May 2020), with 10⁻³ as the e-value cut-off. Arabidopsis thaliana genome accessions (AGIs) of the matched proteins were then submitted to Subcellular Localisation Database For Arabidopsis Proteins 4 (SUBA4) (Hooper et al., 2017) for subcellular location annotation. Differential analyses were performed by Student’s t test using R version 4.0.1. Mean values of protein abundance were used for the calculation of FC. Proteins with a P < 0.05 and an average FC > 1.5 or FC < 0.67 (1/1.5) were considered as DAPs in this study. AGI of DAPs (exclusively present proteins included) were then submitted to David Bioinformatics Resources 6.8 for GO enrichment analysis.

Membrane lipid extraction and targeted HPLC–ESI–MS/MS lipidomic analysis

Membrane lipids were extracted using the protocol described by Barkla et al. (2018) with minor modifications. Five hundred microliters of each FFE sample were aliquoted to a 15-mL falcon tube, to which 1.5 mL of 75% isopropanol with 0.01% butylated hydroxytoluene were added. Samples were snap-frozen in liquid nitrogen after being incubated at 75°C for 15 min in a circulating water bath. After the addition of 750 μL of chloroform and 300 μL of MilliQ water, samples were vortexed and incubated on a rocking shaker for 1 h. Further incubation of 30 min was conducted following the addition of 2-mL 2:1 chloroform: methanol (v/v) to the samples. Following incubation, samples were vortexed and centrifuged at 3,000 rpm for 5 min at 4°C once added with 500-μL 1 M KCl. The upper phase was aspirated, and the lower phase was added with 1 mL of MilliQ. The samples were vortexed and centrifuged at 3,000 rpm for 5 min at 4°C. The upper phase was aspirated, and the lower phase was aliquoted into preweighed 2-mL HPLC glass vials. Samples were weighed following the drying processes using nitrogen gas flow. Lipids were then resuspended by adding 200 μL of butanol: methanol 1:1 (v/v) with 10 mM ammonium formate for HPLC–ESI–MS/MS analysis.

Targeted lipidomic analysis was carried out using an HPLC (Agilent LC 1200) coupled to an Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). For HPLC lipid separation, an Ascentis Express RP Amide column (50 × 2.1 mm 2.7 μm, Supelco, Sigma, St Louis, MO, USA) was used with a water/methanol/tetrahydrofuran gradient. For each lipid sample, 5-μL aliquots were injected. The column temperature was set at 35°C, and the flow rate of the mobile phase was at 0.2 mL/min. The mobile phase was changed from water/methanol/
tetrahydrofuran (50:20:30, v/v/v) to water/methanol/tetrahydrofuran (5:20:75, v/v/v) over a period of 5 min and held for 3 min. Multiple reaction monitoring of the Triple Quadrupole MS/MS was used to identify and quantify glycerolipids from each class. The MS was set at positive mode. About 20–50 compounds were simultaneously measured every 20-ms dwell time. Each chromatographic peak was about 30–45 s wide with a minimum of 12–16 data points collected. The MS parameters were optimized with the capillary voltage at 4,000 V, fragmentor at 140–380 V, collision voltages at 15–60 V, and collision gas (nitrogen) at 7 L/min. Agilent MassHunter quantitative software (version 6; Mulgrave, Australia) was used to process the acquired data.

The lipids were identified using precursor ion or neutral loss. PC and lysoPC (or LPC) ions ([M + H]⁺) were identified by precursor ions of m/z 184.1, PE ions ([M + H]⁺) by neutral loss of 141, PS ions ([M + H]⁺) by neutral loss of 185, PI ions ([M + H]⁺) by neutral loss of 279, PG ions [M + NH₄]⁺ by neutral loss of 189, and PA ions ([M + H]⁺) by neutral loss of 115. NLs were identified based on the precursor ions ([M + NH₄]⁺) and the neutral loss of different FA species. The detected lipids were described as lipid class (the sum of carbon atoms in all the FAs: the sum of double bonds in all the FAs). Lipid standards of PC, PE, PG, PI PS, LPC, LPE, LPG, LPI, TG, and DG were purchased from Avanti Polar Lipids (Alabaster, USA). To prepare the calibration solutions, individual lipid standard solutions were combined and diluted to concentrations ranging from 0.1 to 10 μM. The linear calibration curves were obtained from the chromatographic peak areas of the lipids against their concentrations. Because it was not feasible to acquire commercial lipid standards for every single lipid, the individual lipid peak areas relative to the total lipid areas of each replicate were used to carry out the FC analysis. Students t tests and principal component analysis was performed using R.

Data availability
The raw mass spectrometry proteomics data generated in this study have been deposited to Southern Cross University research portal (https://researchportal.scu.edu.au/) and are available under DOI: https://doi.org/10.25918/data.168.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. Scree plot showing the percentage of explained variances of each principal component.

Supplemental Figure S2. Log FC of percent peak area of identified molecular species.

Supplemental Table S1. Characterization of M. crystallinum membrane proteome and its regulations under salt treatment.

Supplemental Table S2. Peak area of identified lipid molecular species normalized to total lipid content.

Supplemental Table S3. Alterations to the lipid metabolism-related proteins.

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