NaCl-promoted Respiration and Cell Division in Halophilism of a Halophyte, the Common Ice Plant Mesembryanthemum crystallinum L.

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NaCl–promoted Respiration and Cell Division in Halophilism of a Halophyte, the Common Ice Plant *Mesembryanthemum crystallinum* L.

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The common ice plant (*Mesembryanthemum crystallinum* L.), a halophytic vegetable plant, shows the maximum growth in the salinity environment where almost all crops die. The NaCl–promoted growth enhancement called halophilism is an essential trait of increased salt tolerance. To elucidate the factors associated with halophilism, we investigated the effects of NaCl on respiration and genes of cell–division–related factors in suspension–cultured cells. Respiration rate increased with the increase of concentration of NaCl, being maximal at 100 mM NaCl. Metabolite analysis showed that contents of metabolites in glycolysis such as glucose, fructose 6–phosphate, and lactic acid were reduced in the NaCl treated cells. The contents of TCA cycle metabolites such as pyruvate (or oxaloacetic acid), citric acid (or isocitric acid), succinic acid, fumaric acid, and malic acid were reduced in NaCl–treated cells at 21 d after treatment, indicating that the metabolic flux of the TCA cycle is activated to increase respiration and production of NADH and $FADH_2$, which are electron donors for ATP synthesis. Expression analysis showed that the expression of eight genes for complex of ETC (electron transport chain complex), ATP synthase, and TCA cycle enzymes was induced in the salt–treated cells within 6–48 h after the onset of treatment. Moreover, we found that among five cell cycle–related genes that we newly annotated, the expression of a gene associated with the progression of the G1 (*McCycD3;1*), a gene for B2–type cyclin–dependent kinase (*McCDKB2;1*), and its cyclin partner forming an active kinase complex in G2/M phase (*McCycD4;1*) were stimulated by NaCl. We suggested that these factors contributed to the enhanced cell growth in the common ice plant halophilism.

Key words: Cell cycle, Common ice plant, Halophilism, Respiration, Salt stress

INTRODUCTION

Soil salinization is an important environmental issue that significantly reduces plant growth and crop productivity. About 10% of the world's total land area (950 Mha) and 50% of all irrigated land (230 Mha) are affected by salinization, leading to massive losses in crop production globally (Shabala, 2013).

Halophytes, which account for approximately 1 – 2% of angiosperm species, can survive and reproduce in saline soils containing salt up to which almost all crops die (Flowers and Colmer, 2008). Some halophytes show maximum growth in saline soil (Flowers and Colmer, 2008). The enhancement of growth by NaCl referred to as halophilism, is an essential trait for adapting to salinity. Many studies on the adaption of halophytes to salinity have shown the physiological traits for survival in high salinity (Flowers and Colmer, 2008; Shabala, 2013; Flowers and Colmer, 2015). However, there have been few studies on the mechanisms of growth promotion under salinity (Himabindu *et al*., 2016; Kaburagi *et al*., 2014; Shabala, 2013; Tran *et al*., 2019, 2020; Yamada *et al*., 2016). Studies on halophilism that have been carried out so far focused on the morphological and physiological characteristics (Wang *et al*., 2012; Lv *et al*., 2012; Kaburagi *et al*., 2014; Yamada *et al*., 2016), and there are few studies on the molecular mechanisms to promote growth in response to NaCl in halophytes.

A halophyte, the common ice plant, *Mesembryanthemum crystallinum* L., exhibits extremely high salt tolerance and has been used as a valuable model for the various studies on salt tolerance (Vera–Estrella *et al*., 1999; Chiang *et al*., 2016). Over recent decades, many studies on physiological, biochemical, and molecular mechanisms of salt tolerance in the ice plant have been reported (Chiang *et al*., 2016). The findings suggest that the ice plant has specific sophisticated mechanisms in response to high saline conditions (Hasegawa *et al*., 2000; Agarie *et al*., 2007) (Niewiadomska *et al*., 2004). However, there had been no reports elucidating the mechanisms of halophilism in the ice plant.

We found previously that NaCl enhanced the growth of ice plants' suspension–cultured cells, and the increased cell growth was attributed to the promoted cell elongation and cell division (Tran *et al*., 2019). Cell

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elongation is the consequence of water incorporation into the cell resulting from the accumulation of osmolytes and ions in the cytosol. It was regulated by the membrane transport system and cell wall structural properties. We found that the accumulation of ions (K^+, NO_3^-, Na^+, and Cl^-) was positively correlated with the growth and the expression of ion homeostasis–related genes for plasma membrane transporters and channels for incorporation increased in the NaCl–growth stimulated cells (Tran *et al*., 2019).

Cell division progresses through four stages of the cell cycle (G1/S/G2/M). Cell division rate is driven by cell cycle regulators such as cyclins (CYCs), cyclin–dependent kinases (CDKs), and CDK inhibitors, which control the checkpoints (e.g., G1/S and G/M checkpoint) and the progression of the phases in the cell cycle (Burssens *et al*., 2000). The cell cycle regulation activity of CDKs, CDK inhibitors, and CYCs is modulated at both transcriptional and post–translational levels. The gene expression of cell cycle regulator genes is associated with specific cell cycle phases (Menges *et al*., 2005). The NaCl stimulated promotion of cell growth (Tran *et al*., 2019) suggests that the cell cycle regulators which promote cell division are activated under favorable NaCl conditions for the ice plant.

The processes of cell division and elongation required ATP to be operated. Thus, ATP synthesis should increase to meet the energy requirement for the NaCl–stimulated growth enhancement. Under salinity conditions, halophytes need ATP for Na^+ and Cl^- sequestration into the vacuole to prevent their toxic effects on cytosolic metabolisms. They also need to maintain active uptake for macronutrient ions and enhance the synthesis of compatible solutes under salt stress conditions for controlling inter–cellular ion homeostasis and osmotic adjustment (Flowers and Colmer, 2008). Thus, the ATP demand in halophytes would considerably increase in salinity conditions (Kumari *et al*., 2015; Yeo, 1983). However, understanding ATP production under salinity has been limited.

In the previous study, we found that ATP synthesis of mitochondria isolated from leaf exhibited an increase up to 34–61% with the increase of salt concentration, indicating that the mechanism of salt–activated mitochondria ATP synthesis may exist in the ice plant (Tran *et al*., 2020). In this study, we investigated the effect of NaCl on the respiratory rate, the content of respiration–related metabolites, and the expression levels of respiration–related genes. Also, we established a cell line suitable for cell–cycle synchronization and investigated the changes in expression levels of cell–cycle–related genes in synchronized cells in response to NaCl.

MATERIALS AND METHODS

Plant growth and NaCl treatments

The ice plant seed was surface–sterilized and germinated in conditions as described previously (Agarie *et al*., 2007). The 14 d old seedling was transplanted to a 3 L pot and grown in a greenhouse for four weeks and daily watered with Otsuka House fertilizer mixture (No.1 and No.2) prepared according to instruction (Otsuka Warehouse Co., Ltd). Salt treatments were carried out with 50, 100, 200, and 400 mM NaCl in the irrigation solution applied to 6 weeks old plants.

Establishment of cell suspension culture and NaCl treatments

Calli were induced from hypocotyls of the 7 d old seedlings for two weeks on a callus induction medium (CIM) containing MS salt and B5 vitamins, 1 mg L^{-1} 2,4–D, 0.2 mg L^{-1} kinetin, 30 g L^{-1} maltose, and 0.8% type A agar, at 25°C in the dark. The calli were subcultured into the fresh CIM every two weeks after initiation unless otherwise indicated. One mL of suspended cells was put into the liquid CIM containing 0 and 100 mM NaCl. The growth was measured as cell volume after sedimentation (CVS) (Mustafa *et al*., 2011). For synchronized cells (see below), 0.5 g of 14 d old cells after onset of subculture was treated with 25 mM NaCl. The dry weight of cells was measured according to Vera–Estrella *et al*. (1999). Packaged cell volume was measured according to Thomas *et al*. (1992).

Measurement of respiration rate

Cells cultured for 23 and 27 weeks were used to measure changes in respiration with time after 100 mM NaCl treatment and response to different NaCl concentrations, respectively. One mL of suspended cells was taken from the culture flask and placed in the electrode chamber (CB1–D3, Hansatech) containing the culture medium. Respiration rate was measured using a liquid phase oxygen electrode with a Clark–type polarographic oxygen electrode (CB1–D3, Hansatech) in the dark at 25°C. NaCl concentration in the solution was adjusted to 0, 25, 50, 100, 200, and 400 mM.

Metabolite analysis

Suspension cultured cells treated with 100 mM NaCl and without NaCl for 21 and 28 days were filtered through Whatman qualitative filter paper No.1 to collect cells. The cells were dried at 80°C for 24 hours. 15 mg of the ground sample was mixed with a solution containing the standard ribitol (ethanol: ultrapure water: chloroform = 5:2:2) by heating and shaking at 1200 rpm for 30 minutes at 37°C. The supernatant was obtained. After drying, the extract was dissolved in methoxyamine hydrochloride pyridine solution and oxidized by heating and shaking at 1200 rpm at 30°C for 90 min. N–methyl–N–trimethylsilyl–trifluoroacetamide (MSTFA) was also added to the extract, and the extract was trimethylsilylated (TMS) by heating and shaking at 1200 rpm for 30 min at 37°C. The samples were subjected to a gas chromatograph–mass spectrometer (GCMS–QP2010 Ultra, Shimadzu Corporation). The measurement was carried out in the conditions as follows: vaporization chamber temperature: 230°C, ionization voltage: 70 V, injection volume: 1 μl, mass range: m/z 85–500, injection mode: split (1:25), analysis mode: scan, column flow rate: 1.12 ml min^{-1} (He) (linear flow rate), scan speed:

10000 u sec^{-1} (0.05 sec), purge flow rate: 5.0 ml min^{-1}, detector voltage: 0.9 KV, interface temperature: 250°C, solvent cut time: 3 min, ion source temperature: 200°C, data acquisition time: 3.5~24 min, oven temperature: 80°C (2 min)–(15°C min^{-1})–330°C (6 min).

RNA extraction and quantification of relative transcript abundance

Quantitative real–time PCR (qRT–PCR) was performed as previously described by Roeurn *et al*. (2016). The total RNA of the cells was extracted according to the modified SDS/LiCl method (Shirzadegan *et al*., 1991). Genomic DNA was removed using a recombinant DNase I kit (Takara Inc. Japan). cDNA was synthesized from DNase–treated total RNA by reverse transcription reaction with a kit (Rever Tra Ace; Toyobo Ltd., Japan) with oligo $(dT)_{20}$. The relative expression level of the genes was calculated by the ΔΔCT method (Livak and Schmittgen, 2001) after being normalized against the endogenous control. The end–point semi–quantitative PCR (semi–qRT) was performed with a kit (Quick Taq HS DyeMix; Toyobo Ltd., Japan), 0.2 μM specific primers, and 1 ng μL^{-1} the first–strand cDNA. The PCR condition of a thermal cycler (GeneAtlas G–02, ASTEC) was as follows: first denaturation of 2 min at 94°C, followed by 21–29 cycles of 94°C for 30 s, 55–57°C for 30 s, 68°C for 1 min, followed by final elongation of 68°C for 5 min. The *McUBQ* (TC7894) was used as an internal control. The amplified PCR products were electrophoresed on an agarose–solidified gel containing 1.2% agarose powder (Fast Gene, Japan) and 1X TAE (40 mM Tris base, 40 mM glacial acetic acid, and 1 mM EDTA–pH 8.0). The fluorescence intensity emitted from ethidium–bromide–stained bands of PCR products was converted into corresponding values using ImageJ software (https://imagej.nih.gov/ij/). These values were used to calculate the relative transcript abundance of the genes.

Cell cycle synchronization

Callus generated following the third subculture were passed through a stainless–steel sieve with a pore size of 500 μm. One gram of the callus in the filtrate was transferred into a 100 mL flask containing 25 mL of medium CIM (pH 5.7) containing the MS salts, B5 vitamins, 0.2 mg L^{-1} 2,4–D, and 30 g L^{-1} D–maltose, and shook at 125 rpm in the dark at 25°C, for three weeks. From the third subculture onward, 5 mL of the culture after passing through the stainless–steel sieve were cultured in a 100 mL flask containing 20 mL of fresh CIM for two weeks. The subcultures were maintained for 1.5 years before being used for further experiments. After passing through the stainless–steel sieve, the cells in the filtrate were collected using a Whatman No.1 paper filter. To arrest the cell cycle, cells were cultured in the medium without KH_2PO_4 for 72 hours for phosphate starvation (Amino *et al*., 1983). The populations of cells in the M phase in a suspension were defined as the mitosis index (MI) (Polit *et al*., 2012; Kumagai–Sano *et al*., 2006). One gram of the starved cells was put in the CIM (25 mL) containing 25 mM NaCl, and potassium (KH_2PO_4) was added to the solution, and cells were collected at 0, 2, 4, 6, and 8 h after the onset of treatment for analyzing the expression of cell–cycle related genes.

Microscopic observation

Microscopic observation of the size homogeneity of cell clusters in the culture was performed using a microscope (BZ–9000, KEYENCE).

Mitosis index

The mitosis index (MI) was used to estimate the synchrony level of the cell cycle progression after releasing the cells from the KH_2PO_4 starvation. The KH_2PO_4 added cells were collected and washed with PBS buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) (Kumagai–Sano *et al*., 2006). Then, the cells were fixed with Carnoy solution (ethanol: acetic acid at a ratio of 3:1) at least for 1 h. The washed cells with the PBS buffer (Yasuda *et al*., 1988) were stained with 0.5 mL PBS buffer containing 0.5 μg mL^{-1} DAPI (4,6–diamidino–2–phenylindole) and 0.1%Triton–X for 2–5 min. After washing with the PBS buffer, the stained cells were re–suspended in a suitable volume of the PBS buffer before being put onto a slide for counting MI under a fluorescence microscope (BZ–9000, KEYENCE). The cells of the M phase (only in the prophase, anaphase, and metaphase) were counted based on the shape and fluorescent intensity of the nuclei (Polit *et al*. (2012). Counts were made on random transects in three slides (for each biological replication), with 1000 cells counted. MI was calculated as the percent of the cell in the M phase per all counted cells (Kumagai–Sano *et al*., 2006).

Annotation of genes

To annotate the ice plant homologous gene, the ice plant cDNA database, which was constructed by a co–author, Dr. John C. Cushman (University of Nevada, Reno, USA), was used in a BLAST search of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The settings aligned the full–length cDNA sequence of annotated genes with the database of NCBI was also performed to validate their function (Altschul *et al*., 1990; Mount, 2007).

Statistical analysis

BellCurve for Excell (ver 2.15., Social Survey Research Information Co., Ltd.) was used for the statistical analysis.

RESULTS

Growth of plant and cell under salinity condition

Figure 1 shows the growth performance of the ice plant in response to NaCl. The plants grew healthy with vigorous green leaves and stem with NaCl ranging from 50 to 200 mM (Fig. 1, A–E). The growth of plants cultured with 200 mM NaCl was more than 2–fold higher than the untreated control. Many factors influence the growth of the whole plant, such as photosynthesis, partitioning of assimilates to organs, transportation efficiency,

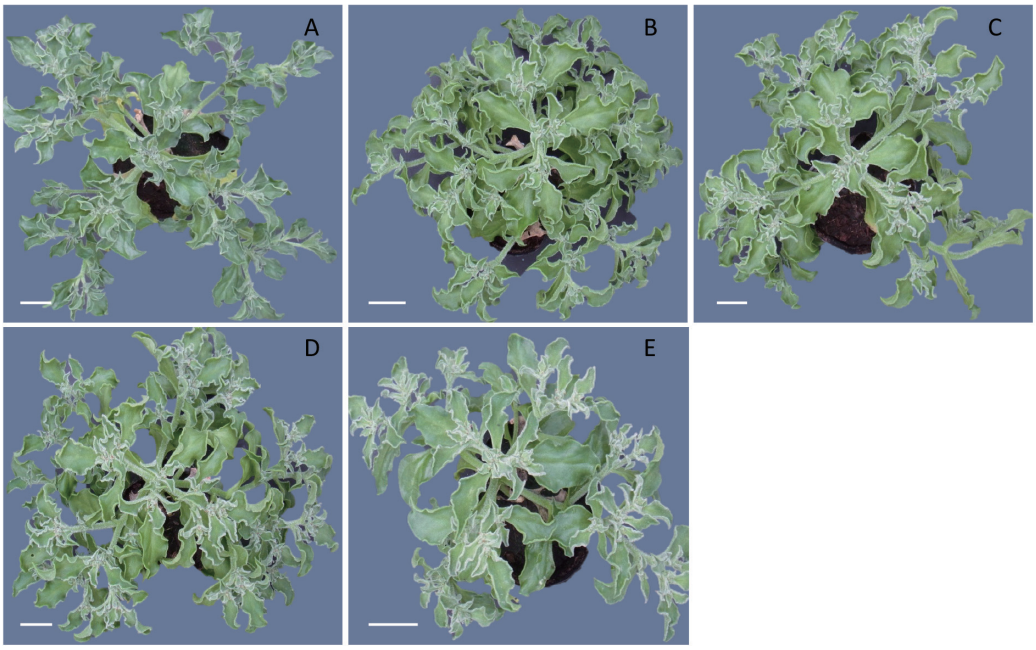

Fig. 1. The growth of the ice plant under different NaCl concentrations. The 6 weeks–old seedlings treated with different concentrations of NaCl. A–E, representative images of the plants grown with 0, 50, 100, 200, and 400 mM NaCl, respectively. Bar = 5 cm.

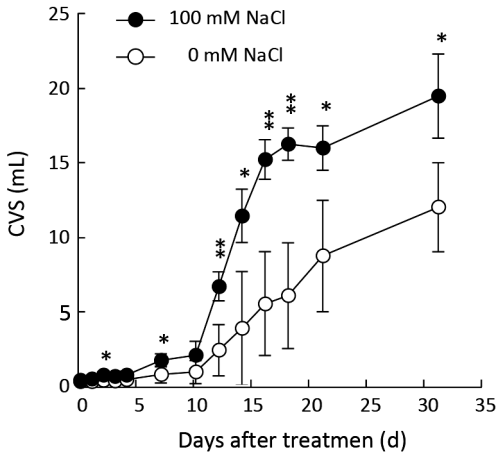

Fig. 2. Changes in the growth of the ice plant's sustention cells cultured with and without NaCl for 31 days without subculture. Data represent mean values ± standard deviations (n=3). Asterisks indicate significant differences by student t–test between NaCl–treated and untreated cells. **P<0.01; *p<0.05.

and respiration. We used suspension–cultured cells to simplify the factors regulating the NaCl–stimulated growth enhancement in the ice plant. In our previous study, the dry weight of the cell increased after 7 d, which cultured in the medium with 50 and 100 mM NaCl and showed maximum growth in the cell growth with 100 mM, and it was inhibited in the medium with 400 mM NaCl compared to the control (Tran *et al.*, 2019). In the present study, we measured the growth performance as cell volume after sedimentation (CVS) (Fig. 2), a good index showing a high positive correlation with fresh weight (Mustafa *et al.*, 2011). The growth evaluated by CVS was enhanced by NaCl, which was also found in the fresh weight, dry weight, and packed cell volume previously (Tran *et al.*, 2019). Growth increased significantly between 10 and 16 days, slowed slightly on 16 days, and gradually increased. The value at 31 days was 1.6 times higher than that of non–salt–treated plants.

Respiration rate

We measured the respiration rate of the cells on days 3, 7, and 14 days after NaCl treatment (Fig. 3). Respiration rate significantly increased at 7 d after the onset of treatment in the cells cultured with 100 mM NaCl (Fig. 3A). When the respiration rate in the cells cultured without NaCl was measured under different NaCl concentrations, the respiration rate increased with increasing NaCl up to 100 mM (Fig. 3B). The respiration rate under 100 mM NaCl was three times higher than that of the untreated control.

Contents of respiration metabolites in NaCl treated cell

Figure 4 shows the contents of respiration–related metabolites treated with NaCl for 21 days and 28 days. At 21 days after NaCl treatment, glucose (A) was slightly reduced to about 86% of the non–salt treated control, and lactic acid (C) was reduced to about 35% of control. At 28 days after treatment, fructose 6–phosphate (B) was reduced to about 43%. Fructose 6–phosphate is phosphorylated by 6–phosphofructokinase and converted to fructose–1,6–bisphosphate. The reaction of fructose 1,6–bisphosphate, the precursor of glyceraldehyde–3–phosphate (GAP), is the rate–limiting reaction

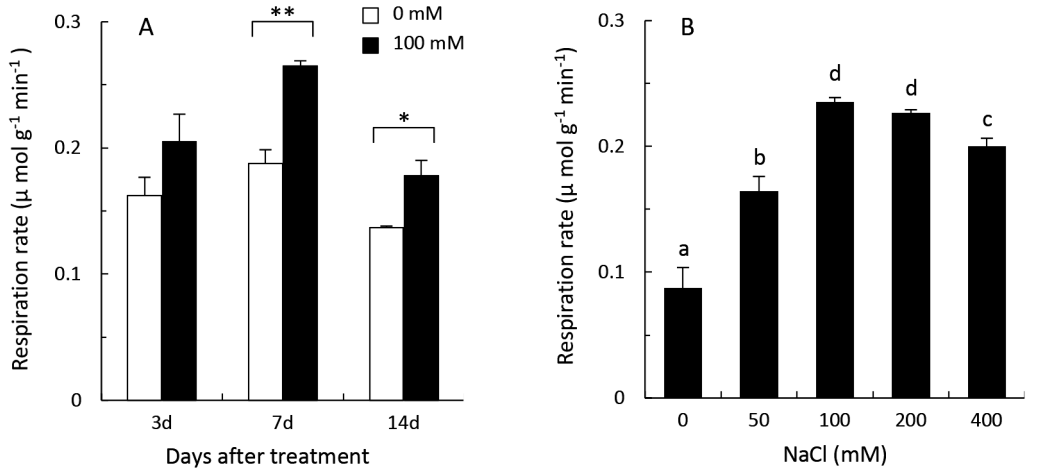

Fig. 3. The effects of NaCl on the respiration rate of the ice plant's cells.
A, respiration rate of cells grown with 100 mM (black) and 0 mM (white) NaCl at different days after treatment. B, respiration rate of the cells cultured without NaCl measured under 0, 50, 100, 200, and 400 mM NaCl. Data represent mean values ± standard deviations (n=3). Asterisks indicate significant differences by student t–test between NaCl–treated and untreated cells. **p<0.01; *P<0.05. Different letters in B indicate significant differences among the treatments at p = 0.05 using the Tukey–Kramer test.

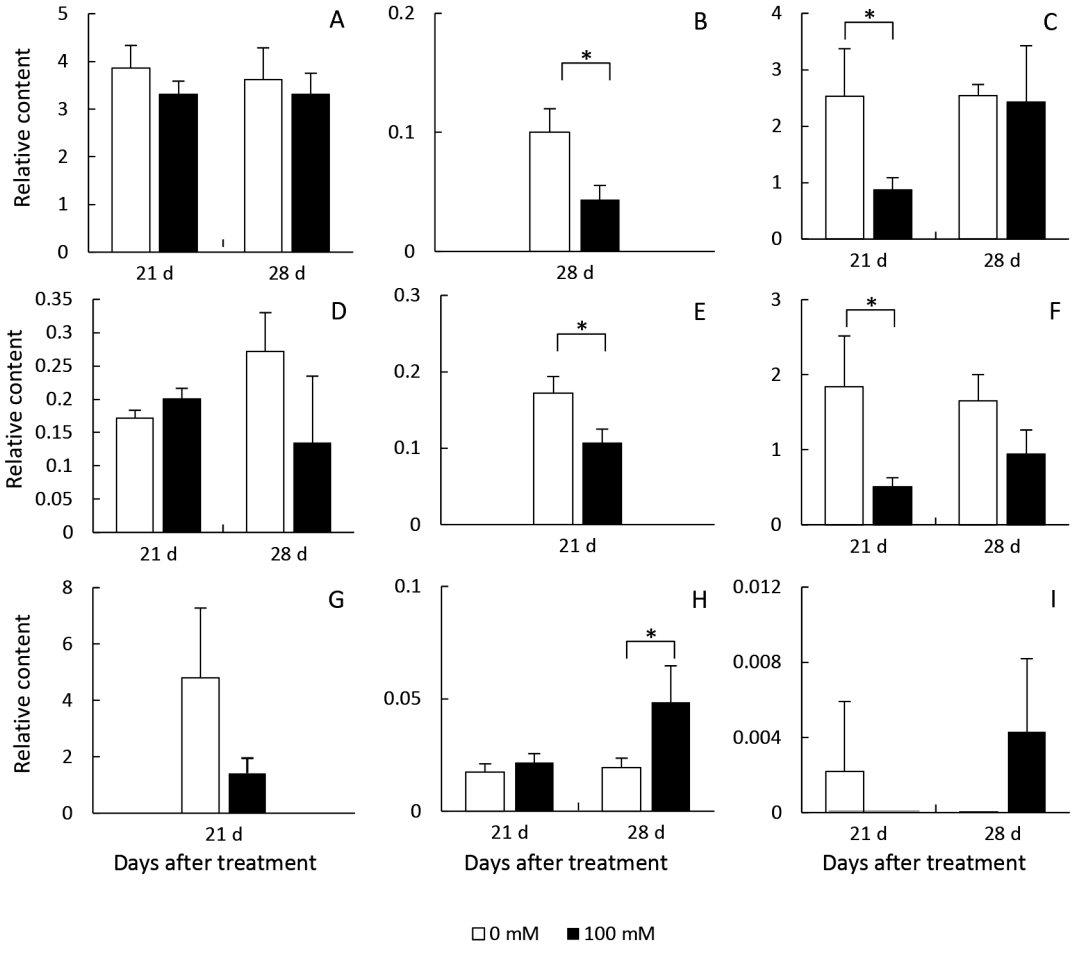

Fig. 4. Metabolites contents of glycolysis and TCA cycle in the cell cultured with and without NaCl. A, glucose; B, fructose–6–phosphate; C, lactic acid; D, pyruvate + oxaloacetic acid; E, aconitic acid; F, citric acid + isocitric acid; G, succinic acid (or aldehyde); H, fumaric acid; I, malic acid. The ordinates represent scaled intensity of the relative abundance. Data show mean values ± standard deviations (n=3). Asterisk indicate significant differences by Welch's *t*–test between NaCl–treated and untreated cells (p<0.05).

in glycolysis. The lactic acid is produced by pyruvate oxidation, and NADH is produced due to the lack of NAD^+ in the glycolytic system under low oxygen conditions. These results suggest that in salt–treated cultured cells, glucose was degraded, the progress of respiration suppressed lactate production, and the carbon sources were supplied for the TCA cycle.

Pyruvate, generated in the glycolysis, is converted to acetyl CoA in the TCA cycle. The acetyl CoA binds to oxaloacetate. Then oxaloacetate is consumed to generate CO_2, NADH, and $FADH_2$ in the cycle. Pyruvate (or oxaloacetate) (D) increased by 17% in salt–treated cells at 21 days after treatment compared to the control. In addition, fumaric acid (H) increased by 1.3 times. On the other hand, citric acid (or isocitric acid) (F) decreased to 23.7%. Succinic acid (G) and malic acid (I) decreased to about 29% and 0.4%, respectively. In the salt–treated cells at 28 days after treatment, pyruvate (or oxaloacetate) (D) and citric acid (or isocitric acid) (F) was reduced to 49% and 57% of the control. These results indicated that the metabolic flux of the TCA cycle is activated, and the production of NADH and $FADH_2$ increase, which is required for ATP synthesis.

Annotation and expression analysis of respiration–related genes

Eight genes coding mitochondrial respiration–related proteins were annotated from the cDNA database of the ice plant and NCBI databases based on the sequence similarity with *Arabidopsis* homologs such as a subunit of 400 kDa sub–complex of complex I (*McCI76*), a flavoprotein subunit 1 of complex Ⅱ (*McSDH1–1*), an alternative oxidase 1a (*McAOX1a*), a subunit 7 of complex Ⅲ (*McQCR7*), a subunit 6B of complex IV (*McCOX6B–1*), a beta subunit of ATP synthase (*McATPF1b*), an E1 subunit alpha of pyruvate dehydrogenase (*McPDHE1α*) and malate dehydrogenase (*McmMDH1*) (Table 1). Based on the sequence homology with the reference genes of *Arabidopsis*, the translated amino acid sequence of these genes had high homology (64–94% identity) with the sequence of homologs from other plant species, such as halophyte *Chenopodium quinoa* and *Beta vulgaris* (Table 1).

Effects of NaCl on the expression of mitochondrial respiration–related genes

We analyzed the expression of mitochondrial respiration–related genes in the suspension–cultured cells treated with 100 mM NaCl or untreated control at 6, 12, 24, 48, 168 (7 d), and 336 h (14 d) after the onset of treatment using qRT–PCR. The differences in transcript abundance between the salt–treated and untreated cells were assessed (Fig. 5). The transcript abundance of *McCI76* (A), *McSDH1–1* (B), *McCOX6B–1* (E), *McATPF1b* (F), and *McPDHE1α* (H) were higher in the salt–treated cells within 6–48 h after the onset of treatment than that in the untreated cells. However, there were no significant differences in the transcript abundance of these genes between the salt–treated and untreated cells at 7 and 14 d. The *McQCR7* (D) and *McmMDH1* (G) showed similar expression between the salt–treated and untreated cells at 6, 168 (7 d), and 336 h (14 d) after the onset of treatment, but the transcript abundance of these genes was higher in the salt–treated cells during 24–48 h than that in the untreated cells. Transcript abundance of *McAOX1a* (C) tended to be lower in the salt–treated cells at all times after the onset of treatment than that of the untreated cells.

Establishment of cell lines for cell cycle synchronization

Suspension cultured cells used for cell–cycle synchronization grew vigorously, and their morphological features should be uniform. We obtained a line with faster growth, small homogenized–sized cells suitable for synchronization by subculture passing cells through a sieve for 1.5 years (Fig. 6). Callus cultured for nine weeks formed clusters with an average size of about 330 mm2, whereas cells cultured for 79 weeks had 70 mm2 (Fig. 6A), and the cell suspension was dispersed (Fig. 6B). The shape of the cells three times of passage (9 weeks of culture) was non–uniform, but the size of the cells cultured for a long time (79 weeks) was smaller and more homogeneous in size and with fewer clusters. The growth of cells was most enhanced by 25 mM NaCl. The optimal concentration of NaCl at which the cells showed a maximum reduction from 100–200 mM to 25 mM (Fig. 7A, B). Figure 8 shows the fresh weight (A), dry weight (B), and packed cell volume (PCV) (C)

Table 1. Sequence homology of respiration related genes of the ice plant to homologues from other plant species

Genes	Components	Function	Arabidopsis Locus	Identity (%)	Plant species
McCI76	ETC Complexes	75 kDa subunit of complex I	AT5G37510.1	88.4	*Spinacia oleracea*
McSDH1–1		Flavoprotein subunit of complex Ⅱ	AT5G66760	93.1	*Beta vulgaris*
McAOX1a		Alternative oxidase	AT3G22370	74.8	*Beta vulgaris*
McQCR7		7 subunit of complex Ⅲ	AT4G32470.1	88.1	*Chenopodium quinoa*
McCOX6B–1		6B subunit of complex IV	AT1G22450	64.0	*Corchorus olitorius*
McATPF1b	ATP synthase	Beta subunit of ATP synthase	AT5G08690	88.4	*Solanum lycopersicum*
McmMDH1	TCA cycle enzyme	Malate dehydrogenase	AT1G53240.1	81.8	*Mikania micrantha*
McPDHE1 α		E1 alpha subunit of pyruvate dehydrogenase complex	AT1G59900.1	94.3	*Beta vulgaris*

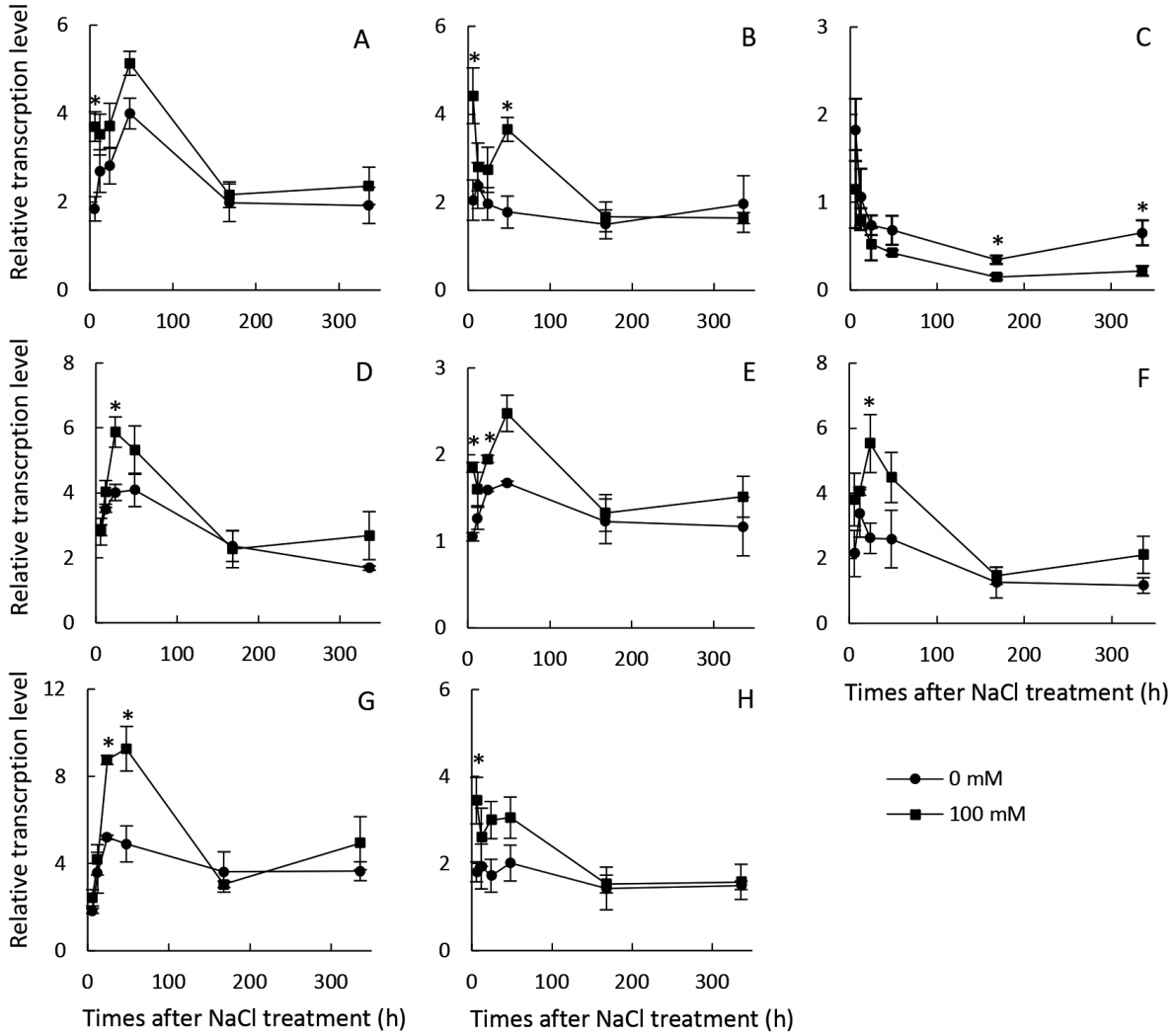

Fig. 5. Expression of respiration related genes in the ice plant's sustention cells cultured with and without NaCl. The suspension cultured cells were collected at six time points [6 h, 12 h, 24 h, 48 h, 168 h (7 d) and 336 h (14 d)]. A, *McCI76*; B, *MsSDH1–1*; C, *McAOX1*; D, *McQCR7*; E, *McCOX6B–1*; F, *McATPF1b*; G, *McmMDH1*; H, *McPDHE1*. Data represent mean value ± standard deviations (n=3). Asterisks indicate significant differences by student *t*–test (P<0.05).

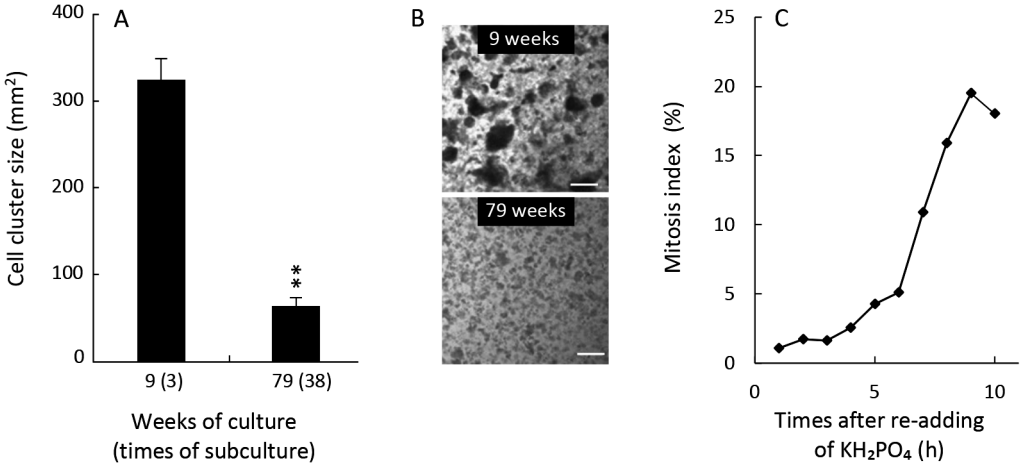

Fig. 6. Characteristics of suspension–cultured cells maintained for 1.5 years used for cell cycle synchronization. A, cell cluster size of suspension cultured cells; B, microscopic images of cells cluster; C, the mitosis index of cell–cycle synchronized cells after re–adding of phosphate. Changes in mitosis index in the cells after being released from the phosphate starvation. Data represent mean values ± standard deviations (n=3). Asterisks in the figure A indicate statistically significant differences in cell cluster size between cells cultured for 9 week and 79 weeks by student t–test between NaCl–treated and untreated cells (p<0.01). Bars in B is 500 μm.

of cells cultured for 3, 6, 9, 12, and 15 days with 25 mM NaCl. Each value significantly increased on the 6 days after NaCl treatment, indicating that the cells still have the traits of halophilism after 1.5 years of subculture.

Annotation of cell cycle–related genes

In the present study, the G1 phase (*McCycD3;1*), S phase (*McHistone H4*), G2 phase (*McKRP3*), and M phase (*McCDKB2;1* and *McCycD4;1*)–related genes were annotated using the ice plant cDNA database and BLAST algorithms. The full–length cDNA sequence of these genes from the cDNA database of the ice plant was for the first time identified, and the BLASTX search in the NCBI database found that the translated amino acid sequence of these genes had high homology (54–96% identity) with that of homologs *Arabidopsis* (Table 2).

Effect of NaCl on the expression of cell–cycle related genes

We analyzed the changes in the expression of annotated genes after NaCl treatment (Fig. 9 and Fig. 10). The differences in the expression levels of *McCycD3;1* and *McCycD4;1* were observed 8 days after treatment between NaCl–treated and untreated cells. They were maintained highly at 8 days after NaCl treatment. On the other hand, no difference was observed in the expression levels of *McKRP3* and *McHistone H4*. We further analyzed the expression of *McCDKB2;1*, which showed the highest scores of total scores, query cover, and identity among the annotated genes (Fig. 10). The relative expression level of *McCDKB2;1* in both NaCl–treated and untreated cells reached a maximum of 4 days after the onset of NaCl treatment. However, it was significantly higher in the NaCl–treated cells than in untreated cells.

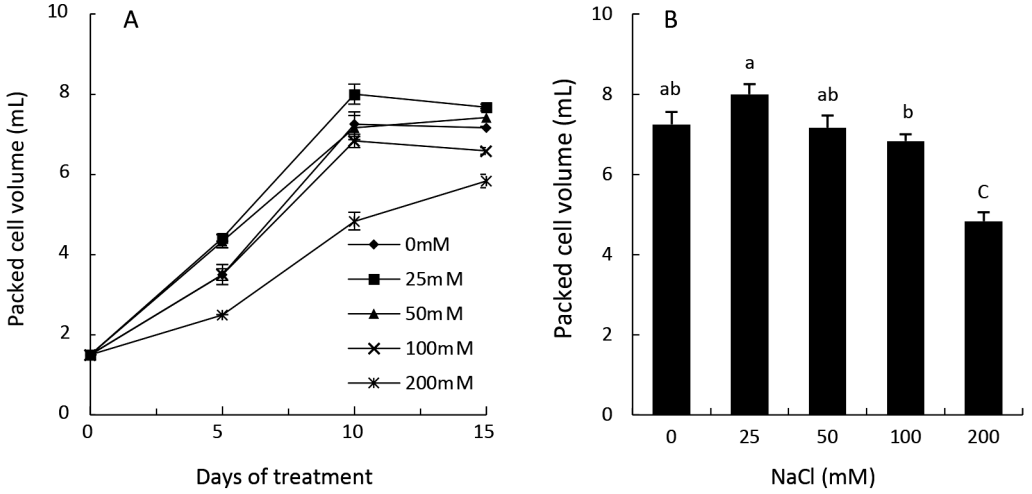

Fig. 7. Growth of suspension–cultured cells maintained for 1.5 years used for cell cycle synchronization under different NaCl conditions. A, changes in growth of cells treated with 0, 25, 50, 100, 200 mM NaCl. B, growth of cells cultured with the same NaCl concentrations as for A at 10 days after treatment. Data represent mean values ± standard deviations (n=3). Different letters indicate significant differences among the treatments ($p<0.05$) using the Tukey–Kramer test.

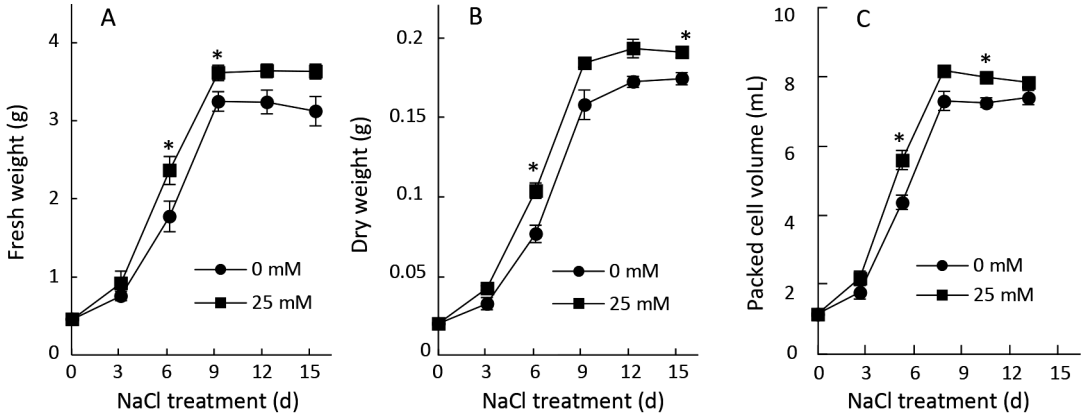

Fig. 8. Changes in the growth the cells maintained for 1.5 years in response to NaCl. A, B, and C, the fresh weight, dry weight, and packed cell volume of cells untreated and treated with 25 mM NaCl were determined at 0, 3, 6, 9, 12, and 15 d after the treatment. Data represent mean values ± standard deviations (n=3). Asterisks indicate statistically significant differences by student *t*–test between corresponding two samples, NaCl–treated and untreated cells ($p<0.05$).

Table 2. Homology of amino acid sequence of the ice plant's cell cycle related genes to homologues from *Arabidopsis*

Genes	Phase	Total Score	Query cover	Identity	*Arabidopsis* Locus
McHistoneH4	S	134	36.0%	96.0%	AT2G28740
McCDKB2;1	G2/M	355	75.0%	80.4%	AT1G76540
McCycD4;1	G2/M	267	43.0%	50.7%	AT5G65420
McCycD3;1	G1/S	321	69.0%	59.7%	AT4G34160
McKRP3	G1/S/G2	123	34.0%	53.5%	AT5G48820

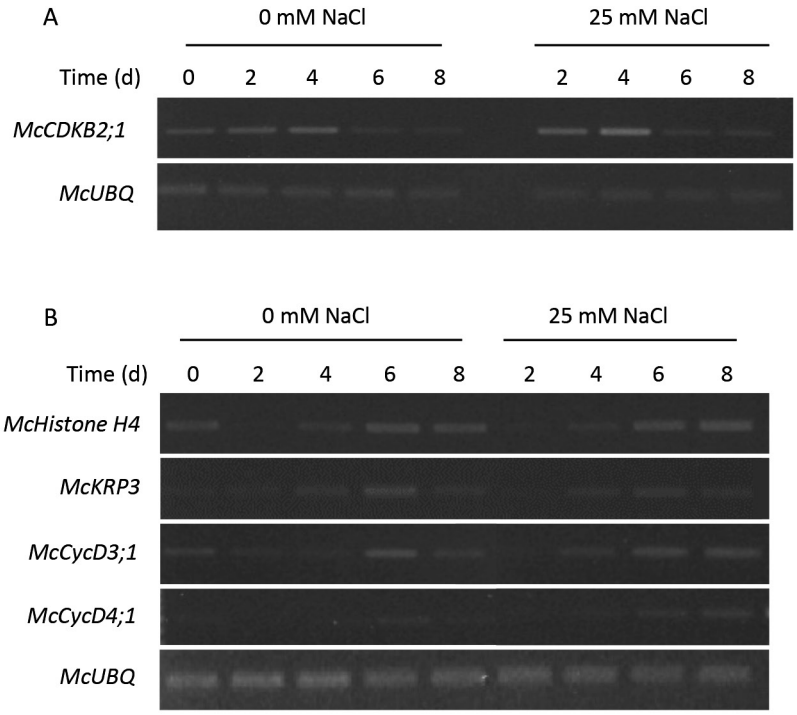

Fig. 9. Representative images of gel electrophoresis of PCR products of cell cycle–related genes. cDNAs were obtained from the cells treated with 25 mM NaCl and untreated control.

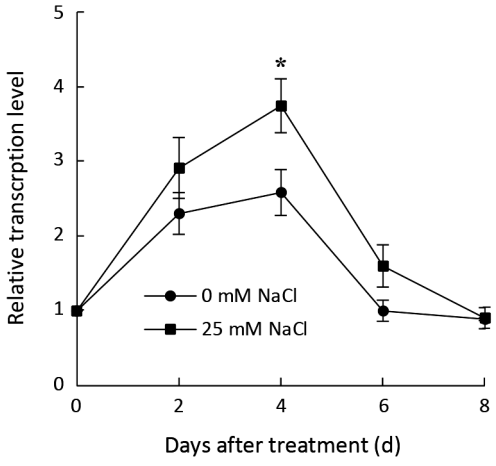

Fig. 10. The expression of *McCDKB2;1* in the NaCl–treated and untreated cells. The gene expression was analyzed using the end–point semi–quantitative PCR (semi–qRT). The relative transcript levels were calculated from signal intensity of PCR products detected using a software ImageJ. Data represented mean values ± standard deviations (n=2). Asterisk indicate significant differences by student–t test in the corresponding two samples ($p<0.05$).

DISCUSSION

NaCl–promoted respiration

Because some energy consumption processes are involved in salt tolerance mechanisms, the ATP synthesis in the mitochondria of halophytes should be promoted to meet the increased energy requirement for the growth enhancement under salinity. We previously found that NaCl enhanced the ATP synthesis in the mitochondria isolated from the leaves (Tran *et al.*, 2020). The NaCl–stimulated increased mitochondrial respiration rate has been reported in some halophyte species and, in other cases, decreased under salt stress conditions (Atreya and Bhargava 2008; Jacoby *et al.*, 2011). In the present study, the respiration rate in the ice plant was higher in the cells cultured with 100 mM NaCl (Fig. 3A). The NaCl–stimulated respiration was also observed in the cells cultured without NaCl. The respiration rate of the cells increased with the increase in the concentration of NaCl, being maximal at 100 mM NaCl (Fig. 3B). This result indicates that NaCl directly influenced the respiration of suspension–cultured cells. Further research is

needed to understand the mechanism for the direct effect of NaCl on mitochondria respiration.

Several proteomic studies on halophytes have suggested that ATP synthesis of mitochondria possibly increased under salt stress due to increased abundances of proteins involved in the glycolysis, TCA cycle, the electron transport chain complexes (ETC), and ATP synthase (Wang *et al*., 2013; Kumari *et al*., 2015). For example, the abundances of subunit beta of mitochondrial ATP synthase, malate dehydrogenase, and succinate dehydrogenase increased in several halophytes under salinity such as *Thellungiella halophila* (Wang *et al*., 2013), *Kandelia candel* (Wang *et al*., 2014), and *Puccinellia tenuiflora* (Yu *et al*., 2011). A combination of transcript and protein abundance studies shows the transcriptional response of mitochondrial functions of plants that rapidly respond to saline conditions (Jacoby *et al*., 2011). We annotated eight genes coding mitochondrial respiration–related proteins from the cDNA database of the ice plant. We found that the expression of *McmATPF1b* significantly increased in the salt–treated cells (Fig. 5F). This result is in line with the increased protein abundance of the beta subunit of mitochondrial ATP synthases observed in halophyte species, such as *Thellungiella halophila* and *Puccinellia tenuiflora* (Yu *et al*., 2011; Wang *et al*., 2013). These results suggest that the mitochondrial ATP synthase of the ice plant was involved in the enhancement of the salt–stimulated ATP synthesis. Also, we found that the expression of genes for the subunits of the four ETC complexes in the salt–treated cells, complex I (*McCI76*), complex Ⅱ (*McSDH1–1*), complex Ⅲ (*McQCR7*), and complex IV (*McCOX6B–1*) (Fig. 5A, B, D, E) increased. These results suggest that the ETC is involved in enhancing the NaCl stimulated ATP production. Other proteomic based investigations on the halophytes species have shown that the activity and abundance of proteins for ETC complexes increased in *Thellungiella halophila* (Zhou *et al*., 2010), *Kandelia candel* (Wang *et al*., 2014), and other halophyte species (Kumari *et al*., 2015). In contrast to the gene expression of the subunits, the expression of *McAOX1a* decreased in the salt–treated cells (Fig. 5C). Alternative oxidases (AOX), final electron acceptors in the alternative respiratory pathway of the ETC in plants, usually is activated to reduce ROS generation when the ETC electron transfer is highly tensed due to the effects of salinity (Saha *et al*., 2016). Thus, the decreased expression of *McAOX1a* indicates the electron transfer efficiency of the ETC in the salt–treated cells, promoting the formation of a proton gradient across the mitochondrial inner membrane. The expression of *McmMDH1* and *McPDHE1α* were increased in the salt–treated cells (Fig. 5G, H), suggesting that the TCA cycle was enhanced in the NaCl treated cells, which contributes to the supply of NADH for promoting the activity of ETC and ATP synthase. A similar tendency was observed for protein abundance of mitochondrial malate dehydrogenases and E1 subunits of pyruvate dehydrogenase in halophyte species grown under salinity, *Physcomitrella patens* (Wang *et al*., 2008) and *Thellungiella halophila* (Wang *et al*., 2013). In summary, the expression of the genes suggested that these genes are involved in the enhancement of NaCl–stimulate promotion of ATP synthesis of mitochondria.

Cell division

Suspensions–cultured cells can be synchronized by release from a specific cell cycle stage, which is imposed either by the depletion of essential growth factors (sugars, phosphate, phytohormone) or by the addition of chemical inhibitors of the cell cycle (aphidicolin, hydroxyurea) (Sala *et al*., 1986). Synchronization of cell suspensions with different treatments has been achieved in various plants such as tobacco (Nagata and Kumagai 1999; Kumagai–Sano *et al*., 2006), *Arabidopsis thaliana* (Zhang *et al*., 2005), and *Catharanthus roseus* (Amino *et al*., 1983; Nishida *et al*., 1992). Suspension cultures of the ice plants have been used for various studies (Vera–Estrella *et al*., 1999), but there has been no report on the synchronized cell cultures. In the present study, we established a suspension cell culture, which showed rapid cell growth rate (Fig. 8) and homogeneous cell clusters (Fig. 6) and found that the phosphate deprivation treatment was effective in arresting the cell cycle of ice plants.

The activity of cell cycle regulatory components such as CDKs, CDK inhibitors, and CYCs are modulated at both transcriptional and post–translational levels. The gene expression of cell cycle regulators is associated with specific cell cycle phases in which the regulators were involved (Menges *et al*., 2005). For example, the expression of *Arabidopsis* Cyc genes (e.g., *CycA3;1*, *CycA3;2*, *CycD2;1*, and *CycD3;1*) appear to be enormously increased with the transition of the cell cycle from G1 to early S phase (Mironov *et al*., 1999; Komaki and Sugimoto 2012), while *Arabidopsis CycBs* transcripts accumulate preferentially at G2 to M transition (Mironov *et al*., 1999). In the S phase, HistonH4 is involved in DNA replication. *Arabidopsis* CDKB1 genes, *CDKB1;1* and *CDKB1;2* accumulate highly from G2 to M phase, and those of the *CDKB2* genes, such as *CDKB2;1* and *CDKB2;2*, are detected more specifically in the M phase (Komaki and Sugimoto 2012). *CycD4* interacts with *CDBK1* to support the G2 to M transition (Kono *et al*., 2003). The transcript level of *Arabidopsis CDK* inhibitors such as *KRP1*, *KRP2 and KRP3* strongly increased at different cell cycle phases associated with regulating CDK–CYCs complex activity (Menges *et al*., 2002). The expression regulation of cell cycle–related genes, which encode cell cycle regulators, is considered to drive the cycle progression by altering the phase duration. Accumulating evidence shows that salinity stress delays the progression of the plant cell cycle by modulating the expression of cell cycle–related genes, leading to plants' growth reduction. For example, West *et al*. (2004) found that expression of *CycB1;2* is reduced in primary root tip cells of *Arabidopsis* stressed with 0.5% NaCl. Upon the NaCl treatment, transcript levels of *DC2aAt*, *CycB1;1*, and *CycA2;1* also diminished initially in *Arabidopsis* shoot apex and root tip cells

(Burssens *et al*., 2000). Salt promoting plant growth in the ice plant suggests that cell cycle regulators may be involved in the growth response under NaCl conditions which induce halophylism.

In the present study, we proposed that NaCl might be associated with the expression of CycD3;1 or improve cellular carbohydrate status in the ice plant cells for shortening the G1 phase duration and cell division. Expression analysis of genes in the present study supports this idea (Fig. 9). The expression of *McCycD3;1*, a gene associated with the G1 phase, was maintained more highly in the salt–treated cells than in the untreated, suggesting that NaCl promotes the G1 phase progression. The G1 phase is the duration–adjustable part of the plant cell cycle in response to alternations in environmental conditions, and the G1 progression is transcriptional and controlled by CycDs and CDKA that form heterodimeric protein kinase complex for the transition over the G1/S checkpoint (Riou–Khamlichi *et al*., 2000; Richard *et al*., 2001). The upregulation of *CycD3;1* is directly associated with cellular carbohydrate status (sucrose availability) in *Arabidopsis* cells (Riou–Khamlichi *et al*., 2000).

In contrast to *McCycD3;1*, the expression of S phase–related genes, *McHistone H4*, was not different between the salt–treated and untreated cells (Fig. 9). The *McKRP3*, which encodes a negative inhibitor of the activity of CDKs in the S phase expressed in the salt–treated and untreated cells, suggests that the S phase is not inhibited by NaCl (Richard *et al*., 2001; Beemster *et al*., 2003). The gene *McCDKB2:1* would drive the M phase in the ice plant. The relative expression level of *McCDKB2:1* in both NaCl–treated and untreated cells reached a maximum level on the fourth day after the onset of NaCl treatment. However, it was significantly higher in the NaCl–treated cells than in the untreated cells (Fig. 10), indicating that the increased expression of cyclin–dependent kinase may be attributed to the NaCl–promoted cell division. The relative expression level of *McCycD4;1* was maintained in NaCl–treated cells. The *CycD4;1* is a novel cyclin partner of B2–type cyclin–dependent kinase (CDKB) and forms an active kinase complex in the G2/M phase (Kono *et al*., 2003). Therefore, *McCycD4;1* may form a complex with *McCDKB2;1* and is associated with G2 to M transition. These genes would be contributed to the halophilism, the NaCl promoting cell growth, through activated cell division in the ice plant.

AUTHOR CONTRIBUTIONS

R. Sato carried out the cell culture experiment and data analysis of the metabolome and drafted the manuscript. Dr. D. Q. Tran carried out the sequence alignment and the expression analysis for respiration– and cell cycle–related genes. K. Yoshida and Jian Dang performed the experiments on respiration. A. Konishi carried out the experiments on mitochondria isolation and ATP synthesis. Dr. S. Ohnishi performed metabolome analysis. Dr. J. C. Cushman provided cDNA libraries. Dr. S. Agarie conceived the study, participated in its design and coordination, and helped to draft the manuscript.

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