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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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Name

学位論文題目： Studies on the halophilism in a halophyte, the common ice plant, *Mesembryanthemum crystallinum* L.
Title of Dissertation (塩生植物アイスプラントの好塩性に関する研究)

学位論文要約：
Dissertation Summary

Salinity, which is caused by predominant presence of NaCl in soils, is known as a serious environmental problem that reduces plant growth and agricultural productivity (Flowers and Colmer 2008; Panta et al. 2014). About 50% of total cultivable land (ca. 230 million ha) are affected by salinity, and the proportion of salinized land areas is increasing (Ruan et al. 2010; Shabala 2013). Because almost all crops used in agriculture production are sensitive to salinity, improvement of salt tolerance of crops and application of salt tolerant plants as alternative crops are important strategies to maintain stable and sustainable agriculture production (Glenn et al. 1999; Shabala 2013; Panta et al. 2014). Halophytes are considered to be salt tolerant plants that have ability to complete their life cycle under high salinity at which most of crops would die. In addition, some halophytes require salt at some extents for their maximum growth (Greenway and Munns 1980; Flowers and Colmer 2008). The promotion of growth by salt is referred to as halophilism or salt-loving trait, which is an important trait for the adaptation of halophytes to salinity. Elucidating mechanisms of the halophilism can provide valuable mechanistic insights in characteristics required to improve salt tolerance in crops (Kawana and Sasamoto 2008; Shabala 2013; Himabindu et al. 2016; Yamada et al. 2016). However, there have still been few studies on the halophilism, and previous reports have mainly focused on growth performance, morphological and physiological responses (Mori et al. 2006; Lv et al. 2012; Wang et al. 2012; Kaburagi et al. 2014; Yi et al. 2014; Yamada et al. 2015; Konishi 2016; Yamada et al. 2016), and the detail mechanisms remain to be elucidated.

The common ice plant (*Mesembryanthemum crystallinum* L.), a facultative halophyte native to South and Eastern Africa, can tolerate high salinity with NaCl concentration equivalent to that of seawater (Bohnert et al. 1988; Adams et al. 1998). The growth promotion of the ice plant is observed under saline conditions containing some amounts of NaCl up to ca. 200 mM (Flowers et al. 1977; Adams et al. 1998; Vera-Estrella et al. 1999; Konishi 2016). The plant has been cultivated as a leaf vegetable (Agarie et al. 2009; Atzori et al. 2017), and also

used as a model system for various studies on salt tolerance (Bohnert et al. 1988; Adams et al. 1998; Bohnert and Cushman 2000; Chiang et al. 2016; Hong et al. 2019). Although many mechanisms of salt tolerance in the ice plant have been well-known, mechanisms of the halophilism are still unclear. In a study in our laboratory, Konishi (2016) showed the growth enhancement of suspension-cultured cells of the ice plant with 25-100 mM NaCl, and the growth enhancement was attributed to the increase of cell size and cell number. In addition, the increased accumulation of ions K^+, Na^+, NO_3^-, and Cl^- was observed in the cells grown under favorable NaCl conditions, and ATP synthesis rate was also enhanced in the leaf-derived mitochondria under different NaCl conditions in the assay mixture (Konishi 2016). However, it remains to elucidate mechanisms associated with these responses, by which the halophilism is induced.

Growth of plants is determined by cell division and cell elongation (**Fig. 1**), thus factors involved in these two physiological processes of the growth would contribute to the halophilism. The cell elongation occurs through expansion of cell wall, which results from interrelation between turgor pressure and cell wall elasticity. The turgor pressure is driven by accumulation of cytosolic components (e.g. ions, compatible solutes) and water incorporation (Heyn 1940; Beemster and Baskin 1998). Meanwhile, the cell division is driven by cell-cycle regulators, such as cyclins (Cycs), cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs), which have regulatory roles specific to each phase/checkpoint of the cell cycle (Burssens et al. 2000; Menges et al. 2002). In the processes, the cell elongation and cell division need energy, and ATP synthesis in mitochondria would be required for these functions (**Fig. 1**). Thus, to get insight into mechanisms of the halophilism, the present study aimed to investigate effects of NaCl on factors related to the cell elongation, cell division, and ATP synthesis in the ice plant. The suspension-cultured cells were used as main experimental system in order to simplify the mechanisms.

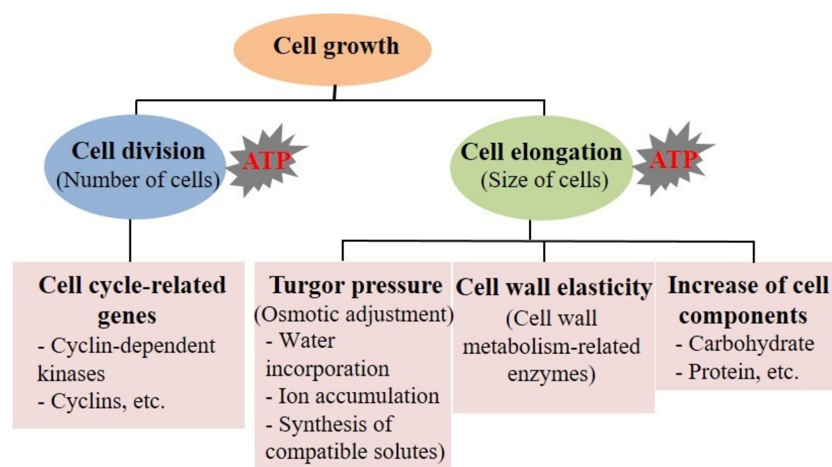


Fig. 1. Conceptual model of factors that regulate cell growth.

The effects of NaCl on growth enhancement and expression of ion homeostasis- and cell wall elasticity-related genes

In the present study, a suspension cell culture of the ice plant as previously described by Konishi (2016), in which the cells showed the maximum growth with 100 mM NaCl, was adopted to analyze on the effects of NaCl on the growth enhancement and expression of ion homeostasis- and cell wall elasticity-related genes. The growth enhancement in the cells treated with 100 mM NaCl occurred following 14 d after the onset of treatment, and it reached a maximum value of about two-fold of that in the untreated cells following 24 d (**Fig. 2**). In addition, under equivalent osmotic pressure generated by NaCl and PEG, the growth enhancement of cells was significantly higher in the culture medium with NaCl than that with PEG (**Fig. 3**), suggesting that NaCl has additional effects, other than osmotic effects, on the growth enhancement.

The increase of cell size and accumulation of Na^+, Cl^-, K^+ and NO_3^- in the ice plant cells grown with 100 mM NaCl (Konishi 2016) indicate that the cells may regulate the ion homeostasis and osmotic adjustment for the cell elongation (**Fig. 2**). In the present study, it was suggested that the cells grown with NaCl incorporates Na^+ and Cl^- into the cytoplasm to increase osmotic pressure, and the excess Na^+ and Cl^- in the cytoplasm are sequestrated into the vacuole depending upon a proton gradient generated by the V-ATPase. Due to the decrease of osmotic potential in the vacuole, compatible solutes (e.g. proline, pinitol, and ononitol) are synthesized for osmotic adjustment. As a consequence, it increases the osmotic pressure and leads to water incorporation. The water influx increases turgor pressure and induces cell wall elasticity, and thus the cells elongate. During the process, the cells are able to actively absorb essential nutrients such as K^+ and NO_3^- to maintain the homeostasis of these ions for the growth. The sequential reactions might form the mechanism of the salt-stimulated cell elongation. To elucidate this mechanism and the related factors, expression of genes encoding plasma membrane transporters and channels for incorporation of Na^+ (*McHKT1*) (Su et al. 2003), Cl^- (*McCCC1*), NO_3^- (*McNRT1*) (Popova et al. 2003), K^+ (*McHAK1, McKmt1*) (Su et al. 2001; Su et al. 2002), and water (*McMipC*) (Kirch et al. 2000); tonoplast transporters for vacuolar sequestration of Cl^- (*McCLC1*), Na^+ (*McNHX1*) (Cosentino et al. 2010), and a V-ATPase subunit c (*McVmac1*) (Tsiantis et al. 1996); and enzymes catalyzing synthesis of proline (*McP5CS*) (Oh et al. 2015), ononitol (*McImt1*) (Vernon and Bohnert 1992), and cell wall metabolism (*McXTH*) (Konishi 2016) were examined (**Fig. 4**). The full-length cDNA sequence of *McCLC1* and *McCCC1* were also for the first time identified from a cDNA database of the ice plant, based on the sequence similarity to that of the homologous genes from other plant species. Among the genes, the expression of *McHKT1*, *McCCC1*, *McCLC1*, *McNHX1*, *McVmac1*, *McKmt1*, *McNRT1*, *McP5CS*,

and *McImt1* were found to be higher in the salt-treated cells than in the untreated cells at various time points (6 h, 12 h, 24 h, 48 h, 7 d, and 14 d) after the onset of treatment. These expressions were in agreement with the mechanism of salt-stimulated cell elongation, and it also indicated that *McHKT1*, *McCCC1*, *McCLC1*, *McNHX1*, *McVmac1*, *McKmt1*, *McNRT1*, *McP5CS*, and *McImt1* are important factors involved in the ion homeostasis and osmotic adjustment for the halophilism of the ice plant. Meanwhile, the expression of *McHAK1*, *McMipC*, and *McXTH* tended to be unchanged or decreased in the salt-treated cells compared to that in the untreated cells, suggesting that these genes might be not involved in the enhancement of water incorporation and cell wall elasticity in the halophilism (**Fig. 4**).

Establishment of cell cycle synchronization and effects of NaCl on expression of cell cycle-related genes

The increase of cell number in the suspension cell culture with NaCl (Konishi 2016) indicates that the halophilism of the ice plant may involve enhancement of the cell division (**Fig. 1**). In the present study, the effects of NaCl on expression of cell cycle-related genes encoding the cell-cycle regulators were aimed to analyze in order to elucidate the mechanism of salt-stimulated cell division and the related factors. The full-length cDNA sequence of fifteen cell cycle-related genes, which are specific to the G1 phase (*McCycD2;1, McKRP2/ICK2, McCDKA;1, McCycD3;1*), S phase (*McHistone H4, McKRP3, McCKS1At, McE2Fb, McCDKA;1*), G2 phase (*McCDKB1;1, McKRP4, McCycD1;1, McCycB2;1, McCDKA;1*), and M phase (*McCDKB2;2, McCycA2;1, McCycB1;1, McCycD3;1, McCDKA;1, McCKS1At*), were for the first time identified from the cDNA database of the ice plant based on the sequence similarity to that of homologous genes from *Arabidopsis* and other plant species. Cell cycle synchrony of cells is required to observe changes in the expression of cell cycle-related genes during the cell cycle progression (Menges and Murray 2002). Using a suspension cell culture of the ice plant (Konishi 2016) in which the cells showed the growth enhancement by 25 mM NaCl as well as rapid growth and homogeneity in cell clusters (**Fig. 5**), effects of treatments of D-maltose, or K_2HPO_4, or 2,4-D starvation on the cell cycle synchronization were tested. Among the treatments, the K_2HPO_4 starvation for 72 h was the most effective in arresting the cell cycle in the G1 phase. The arrested cells might proceed with the significant synchrony level from the G1 phase to S phase, which was observed through changes in mitosis index (**Fig. 6**) coupled with pattern of expression of *McCycD2;1*, *McHistone H4*, *McCycB2;1* and *McCycB1;1* in the cells after the re-addition of K_2HPO_4 (Menges and Murray 2002). In the asynchronous cells (before the treatment of starvation), the expression of *McCycB2;1*, a marker for active cell division (Hirt et al. 1992), was higher in the salt-treated cells than that in the untreated cells during the exponential phase of the growth cycle (**Fig. 5** and **Fig. 7**), suggesting that NaCl enhances the cell division by

shortening duration of some of the cell cycle phases. In the synchronized cells (**Fig. 6**), the expression of the G1 phase cyclins *McCycD2;1* and *McCycD3;1* increased in the salt-treated cells compared to the untreated cells (**Fig. 8**), suggesting that NaCl promotes progression of the G1 phase for the enhancement of cell division, and that *McCycD2;1* and *McCycD3;1* are involved in the halophilism of the ice plant. Meanwhile, the expression of the S phase regulatory genes, *McHistone H4* and a CDK inhibitor *McKRP3*, were not significantly different between the salt-treated and untreated cells (**Fig. 8**), suggesting that progression of the S phase might be not promoted by NaCl.

NaCl-stimulated ATP synthesis in mitochondria and effects of NaCl on expression of mitochondrial ATP synthesis-related genes

Salinity adaptive responses involved in the salt tolerance and the halophilism contain energy-requiring processes, but mechanism supplying ATP to meet the increased ATP demand remains unclear (Yeo 1983; Flowers et al. 2014; Kumari et al. 2015). In a previous study in our laboratory, the ATP synthesis rate in mitochondria, which were isolated from the ice plant grown under favorable (100 mM) NaCl condition (**Fig. 9**), was found to increase with increasing NaCl levels ranged from 50 to 350 mM in the assay mixtures with the same osmotic condition (2.0 MPa) (**Fig. 10B**) (Konishi 2016). In the present study, the ATP synthesis rate in mitochondria isolated from the plants grown without NaCl and with salt stress of 400 mM NaCl (**Fig. 9**) was also examined in the same assay condition to elucidate the enhancement of ATP synthesis, and the results showed that the ATP synthesis rate was also increased with increasing NaCl levels in the mixture (**Fig. 10A, C**). These results, in combination with that observed by Konishi (2016) (**Fig. 10B**), demonstrated clearly that the ATP synthesis is enhanced by NaCl. The maximum ATP synthesis rate was obtained with 350 mM NaCl, and it was 31-58% higher than that in the mixture without NaCl (**Fig. 10**). However, the enhancement of ATP synthesis in mitochondria differed depending on NaCl conditions of the plant growth (**Fig. 9**). The mitochondria isolated from the plants grown with increasing NaCl concentrations showed the induction of ATP synthesis enhancement at lower NaCl levels in the assay mixtures (**Fig. 10**), suggesting an adaptation mechanism that increases the ATP synthesis in response to NaCl in the plants. To elucidate the mechanism of salt-stimulated ATP synthesis, the present study aimed to examine the effects of NaCl on factors related to the ATP synthesis in mitochondria (**Fig. 11**). The full-length cDNA sequence of genes encoding mitochondrial ATP synthesis–related proteins such as a beta subunit of ATP synthase (*McATPF1b*); subunits of complexes of electron transfer chain (ETC) such as a 76 kDa subunit of complex I (*McCI76*), a flavoprotein subunit of complex II (*McSDH1-1*), a 6B-1 subunit of complex III (*McCOX6B-1*), a subunit 7 of

complex IV (*McQCR7*), and an alternative oxidase (*McAOX1a*); subunits of tricarboxylic acid (TCA) cycle-related enzymes such as an E1 alpha subunit of pyruvate dehydrogenase (*McPDHE1α*) and a malate dehydrogenase (*McmMDH1*); and phosphate transporter (*McMPT1*) were for the first time identified from the cDNA database of the ice plant based on the sequence similarity to that of homologous genes from *Arabidopsis* and other plant species. The expression of *McATPF1b*, *McCOX6B-1*, *McCI76*, *McQCR7*, *McSDH1-1*, *McAOX1a*, *McmMDH1*, *McPDHE1α*, *McMPT1*, and an adenylate transporter *McANT2* were analyzed on the suspension-cultured cells grown without and with 100 mM NaCl (**Fig. 2**) at various time points (6 h, 12 h, 24 h, 48 h, 7 d and 14 d) after the onset of treatment. The expression of *McATPF1b* increased in the salt-treated cells compared to that in the untreated cells, indicating that the mitochondrial ATP synthase is involved in the enhancement of the salt-stimulated ATP synthesis (**Fig. 11**). Also, the expression of *McCI76*, *McSDH1-1*, *McQCR7*, and *McCOX6B-1* increased, but the expression of *McAOX1a* decreased in the salt-treated cells, suggesting that the activity of ETC might be activated by NaCl to enhance proton gradient across mitochondrial intermembrane which induces the ATP synthesis enhancement of ATP synthase (**Fig. 11**). The expression of *McPDHE1α* and *McmMDH1* were higher in the salt-treated cells than that in the untreated cells, suggesting that the metabolisms in TCA cycle for supplying energy-rich substances (NADH and NADPH) to ETC might also be stimulated by NaCl (**Fig. 11**). Also, the expression of *McANT2* increased in the salt-treated cells, indicating that the cell was active for the ATP synthesis as well as the ATP consumption (**Fig. 11**). The expression of *McMPT1* was unchanged between the salt-treated and untreated cells, suggesting that phosphate (Pi) incorporation into mitochondria for the ATP synthesis seems to be transported by other transporters. The expression of *McATPF1b*, *McCI76*, *McSDH1-1*, *McCOX6B-1*, *McQCR7*, *McAOX1a*, *McPDHE1α*, *McmMDH1*, and *McANT2* suggested that the activity of ATP synthase, ETC, TCA cycle might be promoted to enhance the ATP synthesis, and these genes are involved in the enhancement of salt-stimulated ATP synthesis which contributes to the halophilism of the ice plant.

In conclusion, by analyzing involvement of factors related to the cell elongation, cell division, and ATP synthesis, the present study revealed the mechanisms and the factors that contribute to the halophilism of the ice plant. Under favorable NaCl conditions, the ice plant actively accumulates K^+, Na^+, NO_3^-, Cl^-, and compatible solutes to regulate the ion homeostasis and osmotic pressure, which leads to the enhancement of water incorporation and the induction of cell elongation. The expression of ion homeostasis-related genes *McHKT1*, *McCCC1*, *McCLC1*, *McNHX1*, *McVmac1*, *McKmt1*, *McNRT1*, *McP5CS*, and *McImt1* are associated with the salt-stimulated cell elongation. Also, the cell division is enhanced by NaCl through the enhancement of the cell cycle progression. The duration of the G1

phase might be shorten by salt-stimulated expression of *McCycD2;1* and *McCycD3;1*. In addition, to supply energy for the growth enhancement, the ATP synthesis in mitochondria is stimulated. The factors responsible for the ATP synthesis such as the ATP synthase, ETC, TCA cycle might be induced for the ATP synthesis enhancement. *McATPF1b, McCOX6B-1*, *McCI76*, *McQCR7*, *McSDH1-1*, *McAOX1a*, *McPDHE1α*, *McmMDH1*, and *McANT2* are the factors involved the salt-stimulated ATP synthesis.

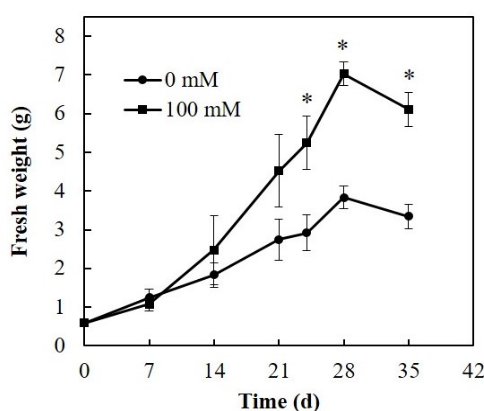


Fig. 2. Effects of NaCl on the growth of suspension-cultured cells. The fresh weight of the cells untreated and treated with 100 mM NaCl were determined at 0, 7, 14, 21, 24, and 28 d after the onset of treatment. Asterisks indicate statistically significant differences between the salt-treated and untreated cells at the same time point by Student's t-test (n=3, $P<0.05$).

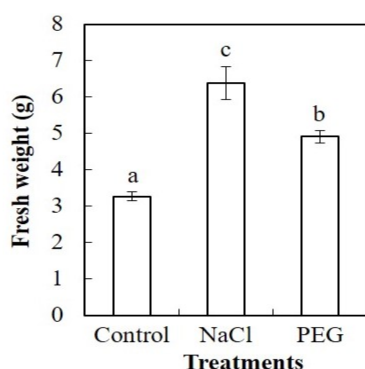


Fig. 3. Effects of NaCl and PEG on the growth of suspension-cultured cells. The fresh weight of the cells untreated and treated with 100 mM NaCl were determined at 24 d after the onset of treatment. Different letters indicate statistically significant differences among the treatments by Turkey-Kramer test (n=3, $P<0.05$).

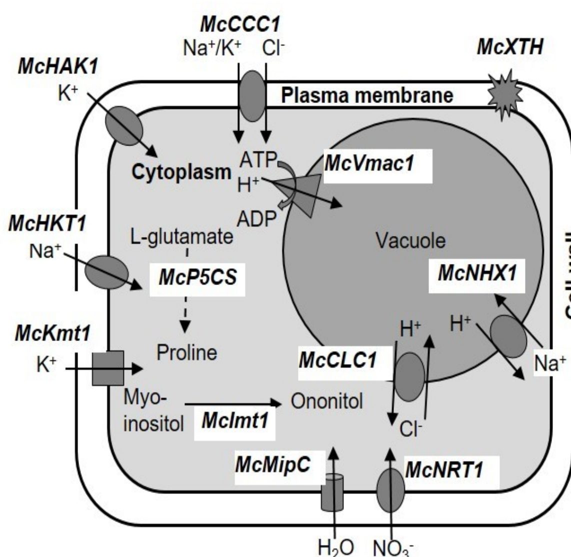


Fig. 4. A proposed model including ion homeostasis- and cell wall elasticity-related factors. Genes are involved in the uptake of Na^+, Cl^-, K^+, NO_3^-, synthesis of proline and ononitol, water incorporation, and cell wall elasticity in the ice plant. *McHKT1,* Na^+ transporter; *McHAK1*, K^+ transporter; *McCCC1*, cation/Cl^- cotransporter; *McKmt1*, K^+ channel; *McNRT1*, NO_3^- transporter; *McNHX1*, Na^+/H^+ antiporter; *McCLC1*, H^+/Cl^-antiporter; *McVmac1*, c subunit of V-ATPase; *McMipC*, water channel; *McXTH*, xyloglucan endotransglucosylase/hydrolase; *McP5CS*, delta 1-pyrroline-5-carboxylate synthase; *McImt1*, myo-inositol O-methyl transferase.

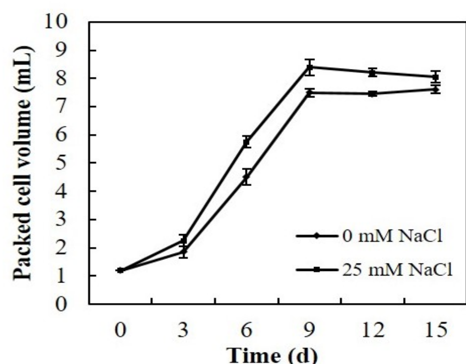


Fig. 5. Effects of NaCl on growth of suspension-cultured cells. Packed cell volume of the cells untreated and treated with 25 mM NaCl were determined at 0, 3, 6, 9, 12, and 15 d after the onset of treatment Data represent mean values ± standard errors (n=3).

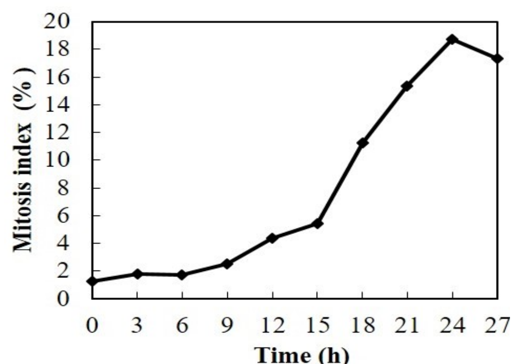


Fig. 6. Cell cycle synchrony in the suspension-cultured cells. Changes in mitosis index were determined at each 3 h interval after being released from the KH_2PO_4 starvation.

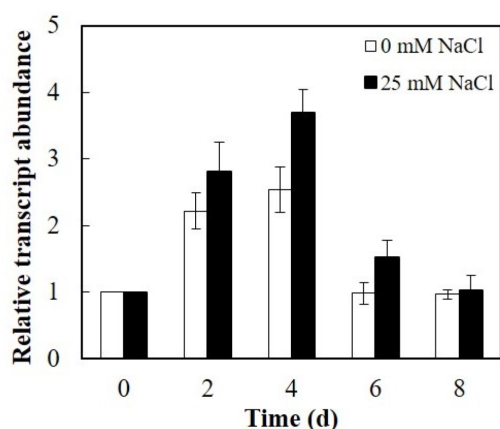


Fig. 7. Effects of NaCl on expression of *McCycB2;1* in the suspension-cultured cells at various time points after the onset of treatment. The relative transcript abundance was determined using endpoint semi-quantitative RT-PCR. Data represent mean values ± standard errors (n=3).

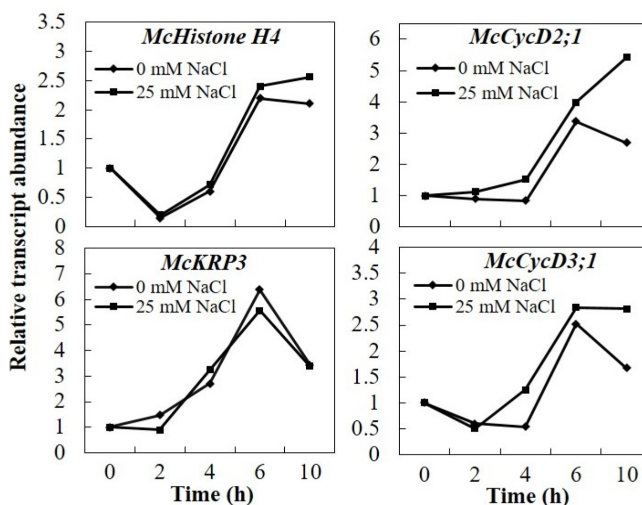
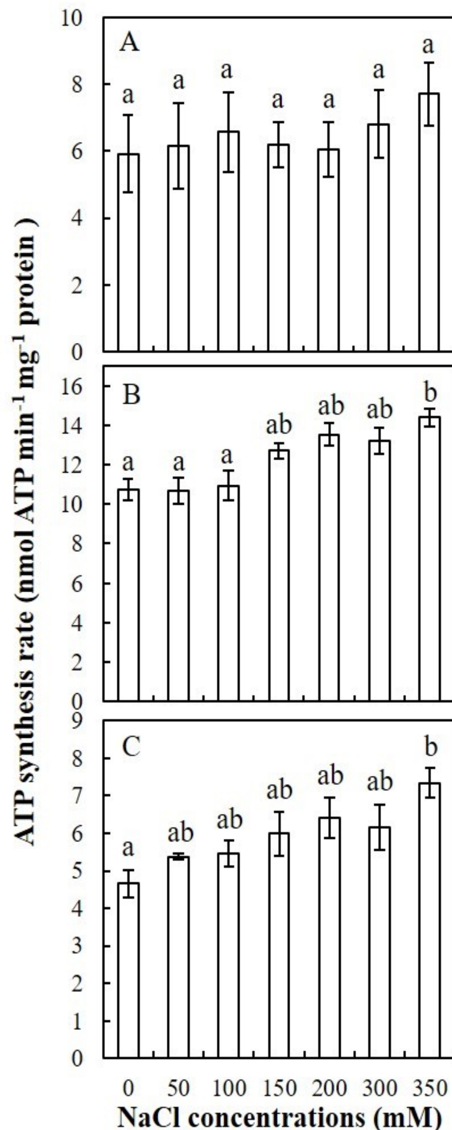


Fig. 8. Effects of NaCl on expression of *McCycD2;1*, *McCycD3;1*, *McHistone H4*, and *McKRP*3 in the synchronized cells at various time points after the onset of treatment. The relative transcript abundance was determined using endpoint semi-quantitative RT-PCR.



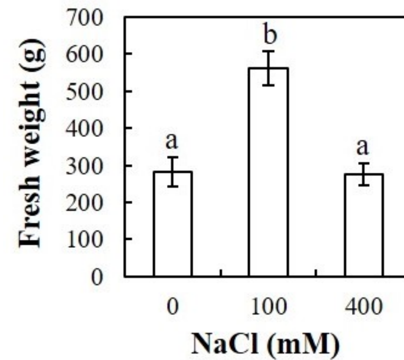


Fig. 9. Effects of NaCl on the growth of shoots of the ice plant. The fresh weight of shoots was observed at 6 weeks after the onset of treatments. Data are mean values ± standard deviations (n=10). Different letters indicate statistically significant difference among NaCl treatments by Turkey-Kramer test (P<0.05).

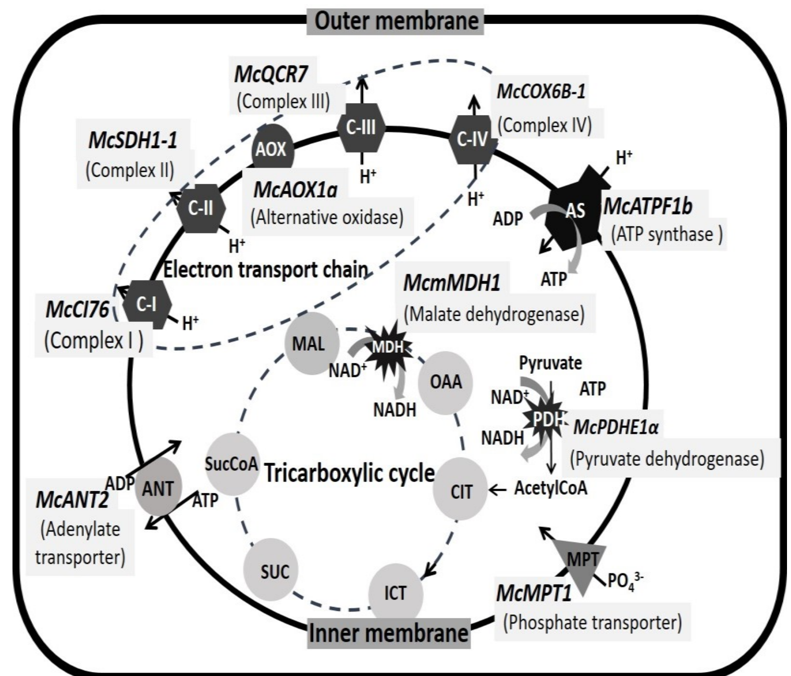


Fig. 10. Effects of NaCl on ATP synthesis rate of isolated mitochondria in the assay mixtures. The mitochondria were isolated from leaves of control plants (A), and plants grown with 100 mM NaCl (B) and 400 mM NaCl (C). The ATP synthesis enhancement of mitochondria isolated from the plant grown with 100 mM NaCl was observed by Konishi (2016). The osmotic pressure of reaction mixtures was adjusted to 2.0 MPa with sorbitol. Data represent mean values ± standard errors (n=3). Different letters indicate statistically significant differences among NaCl treatments by Turkey-Kramer test (P<0.05).

Fig. 11. A proposed model including mitochondrial ATP synthesis related-factors. Genes encode subunits of the ATP synthase, ETC complexes, TCA cycle-related enzymes, and membrane transporters. *McATPF1b*, beta subunit of the ATP synthase; *McCI76*, 76 kD subunit of 400 kDa sub-complex of complex I; *McSDH1-1*, flavoprotein subunit of complex II; *McQCR7*, subunit 7 of complex III; *McCOX6B-1*, 6B-1 subunit of complex IV; *McAOX1a*, alternative oxidase 1a; *McPDHE1α*, E1 alpha subunit of pyruvate dehydrogenase; *McmMDH1*, malate dehydrogenase; *McANT2*, adenylate transporter; *McMPT1*, phosphate transporter.

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（様式５）(Style5)

(注)　要約の文量は，学位論文の文量の約１０分の１として下さい。図表や写真を含めても構いません。

(Note) The Summary should be about 10％ of the entire dissertation and may include illustrations.