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Ion accumulation and expression of ion homeostasis-related genes associated with halophilism, NaCl-promoted growth in a halophyte *Mesembryanthemum crystallinum* L.

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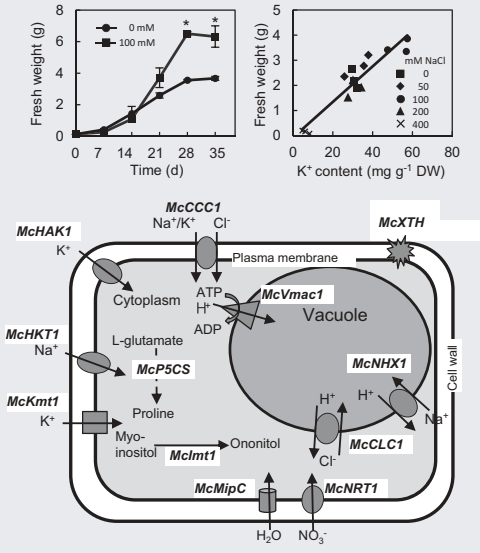
ABSTRACT

A halophyte, the common ice plant (*Mesembryanthemum crystallinum* L.), shows the maximal growth under salinity, in which almost all crops die. The NaCl-stimulated growth, which is referred to as halophilism, is an important trait for adaptation to salinity, but the mechanism is still unclear. To elucidate factors contributing to the halophilism, we tested the effects of NaCl on growth, ion accumulation, and expression of ion homeostasis-related genes in suspension-cultured cells. Among nine ions analyzed, Na^+, Cl^-, K^+, and NO_3^- were accumulated significantly in the cells showing halophilism than that in the salt-untreated cells, and the accumulation of these ions was positively correlated with the growth. Also, the expression of ion homeostasis-related genes for plasma membrane transporters and channels for incorporation of nitrate (*McNRT1*), sodium (*McHKT1*), potassium (*McKmt1*), cations/Cl^- (*McCCC1*), for tonoplast antiporters H^+/Cl^- (*McCLC1*) and Na^+/H^+ (*McNHX1*), and V-ATPase subunit c (*McVmac1*) for sequestration of Cl^- and Na^+ into the vacuole, and for enzymes catalyzing biosynthesis of proline (*McP5CS*) and ononitol (*McImt1*) was higher in the cells showing halophilism than that in the salt-untreated cells. These results indicate that the ion accumulation and the expression of ion homeostasis-related genes contribute to the NaCl-stimulated growth enhancement in the halophyte, the common ice plant.

Abbreviations: CCC: cation/Cl- cotransporter; CLC: H+/Cl–antiporter; DW: dry weight; FW: fresh weight; HAK: high affinity potassium; HKT: high potassium transporter; Imt: myo-inositol O-methyl transferase; MIP: major intrinsic protein; NHX: Sodium/proton antiporter; P5CS: delta 1-pyrroline-5-carboxylate synthase; PCV: Packed cell volume; PEG: polyethylene glycol; XTH: xyloglucan endotransglucosylase/hydrolase

The growth enhancement of the ice plants by NaCl was positively correlated with K^+ accumulation and the increased expression of ion homeostasis related genes contribute to the halophilism.



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Supplemental data for this article can be accessed here.

Introduction

Soil salinization, which is mainly caused by the predominant presence of NaCl, is known as a serious environmental problem that reduces plant growth and agricultural productivity (Flowers & Colmer, 2008). About 10% of the total land area (ca. 950 million ha) and 50% of total irrigated land area (ca. 230 million ha) in the world are affected by salts, and the proportion of salinized land is increasing (Ruan et al., 2010; Shabala, 2013). Because almost all crops 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, Brown & Blumwald, 1999; Panta et al., 2014; Shabala, 2013). Halophytes are salt tolerant plants that have the ability to complete their life cycle under high salinity. Some halophytes also require salt at some extents for the maximum growth (Flowers & Colmer, 2008). The promotion of growth by salt is referred to as halophilism or salt-loving trait, which is an important trait to adapt to saline conditions. The elucidating mechanisms of halophilism can provide valuable mechanistic insights in characteristics required to improve salt tolerance in crops (Himabindu et al., 2016; Shabala, 2013). Although there have been many studies on salt tolerance mechanisms, only a few studies have been carried out on the halophilism, and previous reports have mainly focused on the growth performance and physiological responses at whole plant and organ level (Kaburagi et al., 2014; Lv et al., 2012; Mori, Yoshiba & Tadano, 2006; Nada & Abogadallah, 2015; Wang et al., 2012; Yamada, Kuroda & Fujiyama, 2015, 2016; Yi et al., 2014), and the detail mechanisms remain to be elucidated (Kawana & Sasamoto, 2008; Wang et al., 2012). The growth of plants depends on the capacity of photosynthesis, respiration, translocation, and partition of assimilates to organs. To clarify factors responsible for the growth, the complexity of interactions between organs occurring in intact plant system need to be considered (Gu et al., 2004; Kawana & Sasamoto, 2008). In the present study, to simplify the mechanisms related to the growth enhancement by salt, we used suspension-cultured cells as a model system to analyze growth-related factors influenced by NaCl.

The common ice plant, *Mesembryanthemum crystallinum* L., is a halophyte native to South and Eastern Africa, exhibits tolerance to high salinity in soils containing NaCl at a concentration equivalent to that in seawater (ca. 500 mM) (Adams et al., 1998; Bohnert et al., 1988). The growth enhancement of the ice plant in salinized soils is observed at around 100–200 mM NaCl (Adams et al., 1998; Flowers, Troke & Yeo, 1977). This halophyte has been cultivated as vegetables in some countries (Agarie et al., 2009), and also used as a model system for various studies on salt tolerance (Vera-Estrella et al., 1999. However, there has been no information on the mechanisms of halophilism in this species.

The growth of higher plants is determined by cell division and cell elongation, thus factors involved in these two physiological processes of the growth would contribute to the halophilism. The cell division is regulated by cell-cycle regulators, whereas the cell elongation is determined by incorporation of water, cell wall elasticity, and cytosolic components. An increase in extracellular salt concentration results in changes in intracellular ion homeostasis. Halophytes grown under salinity conditions absorb sodium (Na^+) and chloride (Cl^-) to increase osmotic pressure inside the cells, and the excess Na^+ and Cl^- are sequestrated into vacuole depending upon a proton gradient, which is generated by V-ATPases (Flowers & Colmer, 2008; Flowers et al., 1977; Shabala, 2013). Due to the decrease of osmotic potential in the vacuole, organic compatible solutes such as sugar alcohols, amino acids, betaines, and methylated sulponic compounds are synthesized in cytoplasm for osmotic adjustment. As a consequence, osmotic pressure increases in the cytoplasm and it leads to water incorporation. The water influx increases turgor pressure and cell wall elasticity, which results in cell elongation. During the process, the cells have to maintain homeostasis of essential macronutrients, such as potassium (K^+) and nitrate (NO_3^-), of which the uptake is inhibited in salt-sensitive plant species under high salinity condition. The sequential reactions are crucial for the maintenance of cell growth under salinity, and they should occur in the moderate salinity condition in which the halophilism occurs.

Processes of maintaining ion homeostasis are regulated by transport systems located at plasma and vacuolar membranes. In the ice plant, plasma membrane transporters and channels, such as a HAK-type potassium transporter (McHAK1) (Su, Golldack, Zhao & Bohnert, 2002), Shaker type-potassium channel (McKMT1) (Su et al., 2001), HKT-type transporter (McHKT1) (Su et al., 2003), PTR-type nitrate transporter (McNRT1) (Popova, Dietz & Golldack, 2003), major intrinsic protein (MIP) of water channel (McMIPC) (Kirch et al., 2000), and a tonoplast NHX (Na^+/H^+) antiporter (McNHX1) (Cosentino et al., 2010) and c subunit of V-ATPase (McVmac1) (Golldack & Dietz, 2001; Tsiantis, Bartholomew & Smith, 1996) are considered to be involved in maintenance of homeostasis of K^+, Na^+, NO_3^-, and water incorporation in the plants under salinity. The Cl^- accumulation occurs in the salt-stressed ice plants (Agarie et al., 2007; Wissing & Smith,

2000), but the Cl^- transporters have not been elucidated in the halophyte. In other plants, Cl^- transporters, such as a plasma membrane cation/Cl^- cotransporter (CCC) in *Oryza sativa* (OsCCC1) (Chen et al., 2016; Kong et al., 2011) and a tonoplast H^+/Cl^- antiporter (CLC) in *Glycine max* (GmCLC1) (Wei et al., 2016), are related to Cl^- homeostasis. In the ice plant, proline and ononitol are accumulated in the cytoplasm as compatible solutes in response to salinity (Agarie et al., 2009; Thomas, De Armond & Bohnert, 1992; Vernon & Bohnert, 1992), and genes *McP5CS* and *McImt1* encode enzymes delta 1-pyrroline-5-carboxylate synthase (P5CS) and myo-inositol O-methyl transferase (Imt), which catalyze synthesis of proline and ononitol, respectively (Oh et al., 2015; Vernon & Bohnert, 1992). Moreover, xyloglucan endotransglucosylase/hydrolase (XTH) enzymes, such as a XTH of *Populus euphratica* poplar (PeXTH) (Han et al., 2013), play important roles in the cell wall elasticity in plants under salt stress.

In the present study, we tested the effects of NaCl on growth, accumulation of ions such as K^+, Na^+, NO_3^-, Cl^-, calcium (Ca^{2+}), magnesium (Mg^{2+}), ammonium (NH_4^+), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}), and the expression of genes *McHAK1, McKmt1, McHKT1, McNRT1, McMipC, McNHX1, McVmac1, McCCC1, McCLC1, McP5CS, McImt1*, and *McXTH* in the suspension-cultured cells to elucidate the physiological significance of the ion accumulation and ion homeostasis-related factors in the halophilism of the ice plant.

Material and methods

Plant culture and NaCl treatment

Seeds of *M. crystallinum* L. were surface-sterilized and sowed on germination medium as described by Agarie et al. (2007). Seedlings were transferred to pots and grown in a greenhouse in Kagawa University according to the description by Agarie et al. (2009). NaCl was applied to the 4-week-old plants by irrigation of nutrient solutions 'Otsuka House No. 1 and No. 2' (Otsuka AfriTechno Co. Ltd, Japan) contained 50, 100, 200, and 400 mM NaCl. The fresh weight (FW) of aerial parts was determined at 6 weeks after the onset of NaCl treatment.

Suspension cell culture

Hypocotyls of the 7-d-old seedlings were excised and placed on a callus induction medium (CIM) containing MS salts and B5 vitamins as described by Agarie et al. (2007), 1.0 mg L^{-1} 2,4-D, 0.2 mg L^{-1} kinetin, 7 g L^{-1} agar and 30 g L^{-1} D-maltose (pH 5.7 ± 0.1), and kept in the dark at 25°C. Two weeks after the onset of culture, the calli induced were subcultured into the fresh CIM every 2 weeks. After 2–3 times of subculturing, vigorous and friable calli were passed through a stainless steel sieve with a pore size of 500 μm (Davey & Anthony, 2010), and 1.0 g of the callus in the filtrate was cultured in a 125-mL flask containing 25 mL of the liquid CIM. The flasks containing cells were put on a horizontal-type rotary shaker and continuously agitated at 125 rpm in the dark at 25°C (Thomas et al., 1992; Vera-Estrella et al., 1999).

Salt treatments and measurements of cell growth

Suspension-cultured cells were treated with NaCl by adding NaCl into the culture medium at final concentration of 50, 100, 200, and 400 mM. To test the effect of osmotic pressure on the cell growth, the cells were also treated with a desired concentration of PEG-4000 (PEG) (Cat. 81,240, Sigma Aldrich), which exerted an osmotic pressure equivalent to 100 mM NaCl (Alam, Stuchbury & Naylor, 2002; Money, 1989). The FW, dry weight (DW), and packed cell volume (PCV) of the cells were determined at 0, 7, 14, 21, 28, and 35 d after the onset of treatments using procedures as described in previous reports (Thomas et al., 1992; Vera-Estrella et al., 1999). In the PEG treatment, the cells were collected at 24 d after the onset of treatment by filtration with a Whatman No.1 filter paper, and were washed with 200 mL CIM to remove the PEG residues surrounding the cells prior to the measurement of FW and PCV (Heyser & Nabors, 1981).

Measurement of the number of cells and cell size

The suspension-cultured cells at 0, 7, 14, 21, and 28 d after the onset of treatments were passed through the stainless steel sieve with a pore size of 500 μm. The filtrate was then passed through a 55-μm mesh filter (NITEX, Sefar Co., Switzerland). The number of cells was measured with a cell counter plate (Cat. 177-112C, Watson Co., Ltd, Japan). The cell size was measured with a microscope (BZ-9000, KEYENCE).

Measurement of ion content

The suspension-cultured cells at 28 d after the onset of salt treatment were collected using the method described by Vera-Estrella et al. (1999). Ten milligrams of the dried cells were finely ground with a mortar and pestle, and the powder was mixed with 10 mL of deionized water and kept at room temperature for 24 h (Agarie et al., 2007). The resulting supernatant was filtered through a 20-μm syringe filter, and the measurement of ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, NH_4^+, NO_3^-, Cl^-, SO_4^{2-}, and PO_4^-) was performed using Dionex ICS-900

ion chromatography system (Thermo Fisher Scientific, Waltham, MA, US) (Orsini et al., 2013).

Quantification of transcript abundance

The suspension-cultured cells were collected at 0 h, 6 h, 12 h, 24 h, 48 h, 7 d, and 14 d after the onset of salt treatment. Total RNA was extracted from the cells according to the modified SDS/LiCl method (Shirzadegan, Christie & Seemann, 1991). Genomic DNA was removed using a recombinant DNase I kit (Takara Inc., Japan), and the DNase-treated total RNA was used for cDNA synthesis using Rever Tra Ace kit according to the manufacturer's instruction (Toyobo Ltd., Japan). Real-time PCR for expression analysis of genes was performed with conditions as described by Roeurn et al. (2016). Primer pairs for the amplification of genes such as *McHAK1* (GenBank accession number (GB No.), AF367864), *McKmt1* (GB No., AF267754), *McHKT1* (GB No., AF367366), *McVmac1* (GB No., X94999), *McNRT1* (GB No., AA749492), *McMipC* (GB No., U73466), *McNHX1* (GB No., AM746985), *McP5CS* (GB No., AF067967.1), *McImt1* (GB No., M87340.1), *McUBQ* (GB No., TC7894), *McXTH, McCLC1*, and *McCCC1* were designed using the Primer3 software (*Supplementary Table 1*). In the process, the full-length cDNA of *McXTH, McCLC1*, and *McCCC1* were newly identified from the cDNA database of the ice plant which constructed by the laboratory of Dr. John C. Cushman (University of Nevada, Reno, USA) using BLAST search algorithms (Altschul et al., 1997) against the reference genes such as *PtXTH* (GB No., XM_002318900), *GmCLC1* (GB No., AY972079.1), and *OsCCC1* (GB No., GU250733.1). The relative transcript abundance was calculated on the basis of $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), which was normalized to the transcript level at 0 h. The relative transcript abundance of *McImt1* in the salt-treated cells at 48 h was normalized to the transcript level of the untreated cells. The *McUBQ* was used as an endogenous control.

Results

Growth of shoot and suspension-cultured cells under different NaCl conditions

The FW of shoot of the plants grown with NaCl increased with the increase of NaCl concentration at 50, 100 and 200 mM, and it was about 2-fold greater than that of the plants grown without or with 400 mM NaCl (Figure 1(a)). There was no significant difference in the shoot FW of the plants grown with 50, 100, and 200 mM NaCl (Figure 1(a)). The FW, DW, and PCV of the suspension-cultured cells treated with NaCl for 28 d at the saturation phase of growth cycle (Figure 2(a,b)) increased with 50 and 100 mM NaCl, but they decreased with 200 and 400 mM NaCl (Figure 1(b–d)). The maximum growth enhancement was observed from the cells treated with 100 mM NaCl (Figure 1(b,c)). The enhancement of FW and DW in the cells treated with 100 mM NaCl occurred following 14 d after the onset of treatment, and it reached a maximum value, which was about 1.6- and 1.7-fold of that in the untreated cells at 24 d (data not shown) and 28 d, respectively (Figure 2). The PCV and FW of the cells treated with PEG or NaCl for 24 d were about 1.2 and 1.4 or 1.5 and 1.9 fold higher than those of the untreated cells, respectively (Figure 2(c,d)). The PCV and FW of the salt-treated cells were significantly higher than those of the PEG-treated

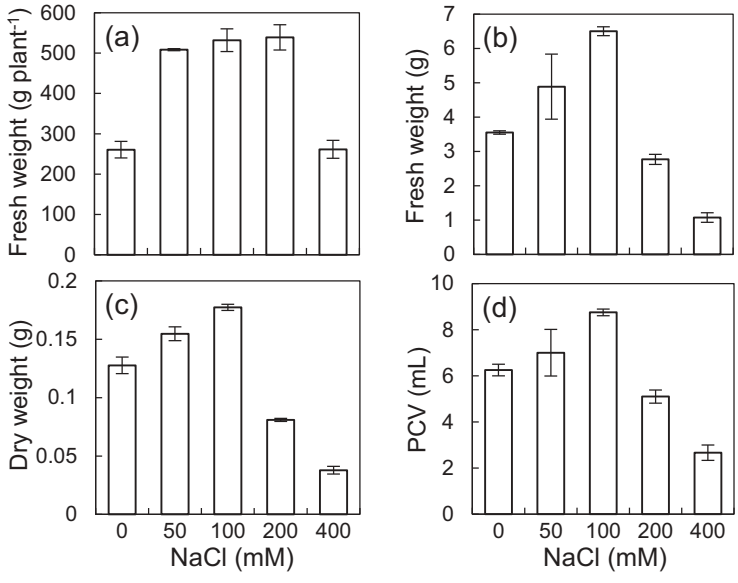

Figure 1. Effects of NaCl on the growth of shoot and suspension-cultured cells in the ice plant.

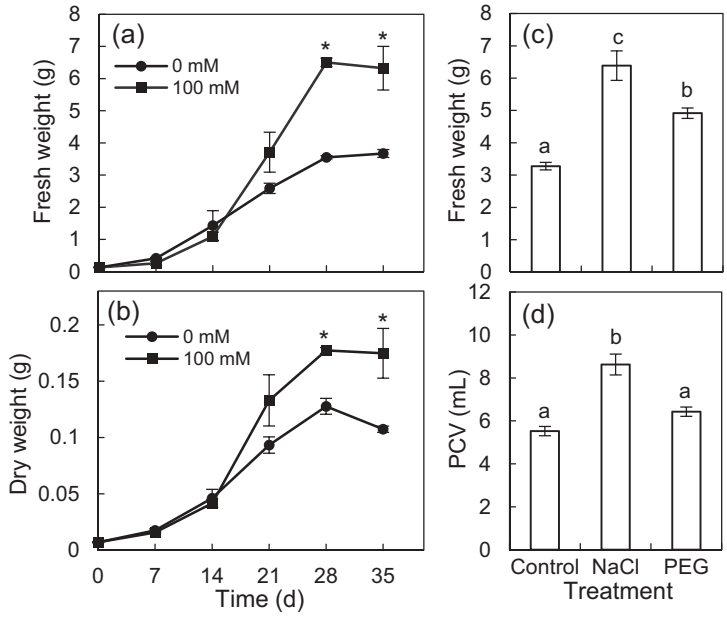

Figure 2. Effects of NaCl and PEG on the growth of suspension-cultured cells.

cells (Figure 2(c,d)). The number of cells (Figure 3(a)) and cell size (Figure 3(b)) tended to increase in the salt-treated cells compared to the untreated cells following 14 d after the onset of treatment.

Ion accumulation in the suspension-cultured cells

The content of PO_4^-, SO_4^{2-}, NH_4^+, Mg^{2+}, and Ca^{2+} in the cells at 28 d after the onset of salt treatments tended to decrease or maintain with increasing NaCl concentrations (Figure 4(a)). Whereas, the content of K^+, Na^+, NO_3^-, and Cl^- increased with increasing concentration of NaCl up to 100 mM (Figure 4(b)). The contents of K^+ and NO_3^- decreased, the contents of Cl^- unchanged, and the contents of Na^+ increased under 200 and 400 mM NaCl (Figure 4(b)).

Correlation between the growth of cells and ion accumulations

The FW of cells treated with NaCl was positively correlated with the content of Na^+ (r = 0.866) and Cl^- (r = 0.902), except that the cells were treated with 200 and 400 mM NaCl (Figure 5(a,b)). Between the FW and the content of K^+ in the cells treated with NaCl showed a strongly positive correlation (r = 0.942) (Figure 5(c)). Also, there was a positive correlation between the FW of cells and the content of NO_3^- (r = 0.654), except that the cells were treated with 400 mM NaCl (Figure 5(d)).

Expression of ion homeostasis-related genes

The full-length cDNA sequences of the *McCLC1*, *McCCC1*, and *McXTH* were identified for the first time from the cDNA database of the ice plant established by the laboratory of Dr. John C. Cushman using the BLAST search algorithms (Altschul et al., 1997). The sequence similarity of *McCLC1*, *McCCC1*, and *McXTH* to sequences of functionally known reference genes from soybean *GmCLC1*, rice *OsCCC1* and poplar *PtXTH* was 74%, 73%, and 74%, respectively (Table 1). The change in the relative transcript abundance of *McHKT1*, *McCCC1*, *McCLC1*, *McNHX1*, *McHAK1*, *McVmac1*, *McKmt1*, *McNRT1*, *McP5CS*, *McImt1*, *McMipC*, and *McXTH* in the cells untreated and treated with 100 mM NaCl was observed at 6 h, 12 h, 24 h, 48 h, 7 d, and 14 d after the onset of treatment (Figure 6).

Genes related to the incorporation of Na^+ and Cl^-

The transcript abundance of *McHKT1* increased transiently in the salt-treated cells at 6 and 48 h after the onset of treatment compared with that in the untreated cells, although the statistical significance (P < 0.05) was not observed at 48 h (Figure 6(a)). Meanwhile, the *McCCC1* was expressed with significantly higher levels in the salt-treated cells during 6–24 h after the onset of treatment (Figure 6(b)).

Genes related to sequestration of Na^+ and Cl^- into the vacuole

The transcript abundance of *McNHX1* gradually increased in the salt-treated cells and statistically significances

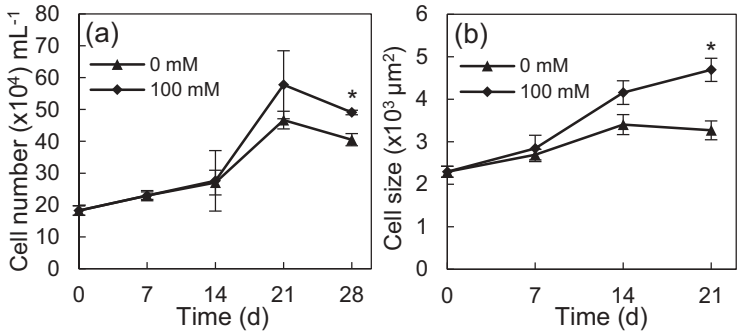

Figure 3. The number of cells and cell size in the suspension cell cultures with NaCl.

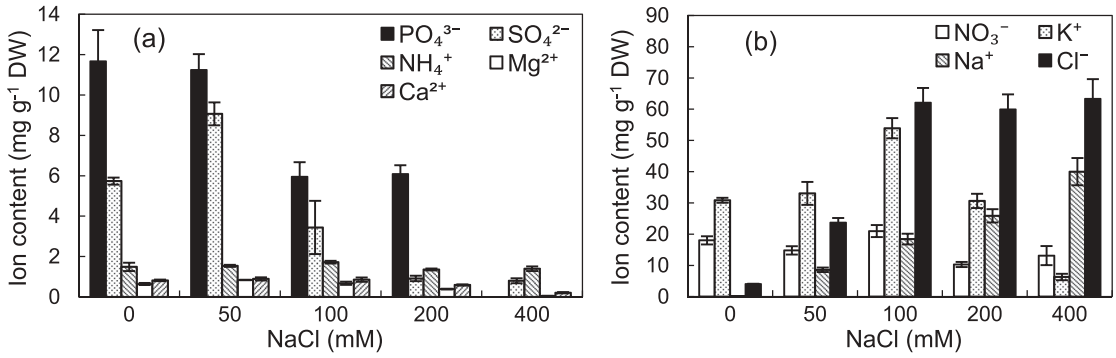

Figure 4. Content of ions in suspension-cultured cells treated with different NaCl.

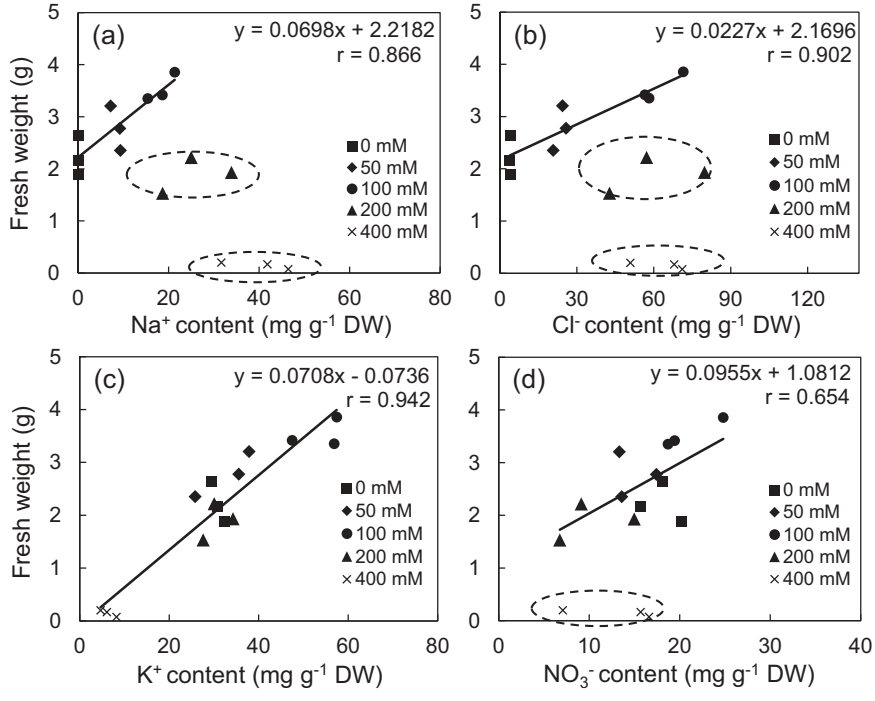

Figure 5. Correlation between the ion content and the fresh weight of cells grown under different NaCl.

Table 1. Sequence homolgy of putative orthologues of the ice plant to that of reference genes for CLC, CCC, and XTH from other plants.

Reference genes (GB No.)	Function	Putative orthologues in the ice plant[a]	Homology	Plant species
GmCLC1 (AY972079.1)	Tonoplast Cl^-/H^+ antiporter	*McCLC1*	74%	Soybean
OsCCC1 (GU250733.)	Plasma membrane cation/Cl^- cotransporter	*McCCC1*	73%	Rice
PtXTH (XM_002318900)	Cell wall xyloglucan endotransglucosylase/hydrolase	*McXTH*	74%	Poplar

[a] The cDNA sequences of *McCLC1*, *McCCC1*, and *McXTH*, which were identified from the cDNA database of the ice plant using BLAST search algorithm against the reference genes.

($P < 0.05$) were observed at 14 d after the onset of treatment (Figure 6(c)). Meanwhile, the expression of *McCLC1* significantly increased in the salt-treated cells at all of the time points except at 48 h (Figure 6(d)). The transcript abundance of *McVmac1* was significantly higher in the salt-treated cells during 12 h–14 d after the onset of treatment, except at 7 d, than that in the untreated cells (Figure 6(e)).

Genes related to the homeostasis of K^+ and NO_3^-

The transcript abundance of *McNRT1* was higher significantly in the salt-treated cells after the onset of treatment except at 7 d (Figure 6(f)). Meanwhile, the expression of *McKmt1* was higher in the salt-treated cells at 6 and 12 h, although the statistical significance ($P < 0.05$) was observed only at 6 h, and it decreased with the lapse of time (Figure 6(g)). There was no significant difference in transcript abundances of *McHAK1* between the salt-treated and untreated cells (Figure 6(h)).

Genes related to the synthesis of proline and ononitol

Osmotic adjustment in the cytoplasm of the salt-treated cells can be achieved by the synthesis of compatible solutes such as proline and ononitol, which were regulated by the expression of *McP5CS* and *McImt1*. The *McP5CS* expressed with significantly higher levels in the salt-treated cells after the onset of treatment than that in the untreated cells, the statistical significances ($P < 0.05$) was observed at 24 h, 48 h, and 7 d (Figure 6(i)). The transcript abundance of *McImt1* in the cells was undetectable at all of the time points except at 48 h. The expression of *McImt1* at 48 h was about 2-fold higher in the salt-treated cells than that in the untreated cells (Figure 6(j)).

Genes related to water incorporation and cell wall elasticity

The transcript abundance of *McMipC* was lower in the salt-treated cells during the time tested compared to that in the untreated cells. The statistical significance between the salt-treated and untreated cells was observed at 12 and 24 h (Figure 6(k)). The expression of *McXTH* was lower at 7 d in the salt-treated cells than that in the untreated cells (Figure 6(l)).

Discussion

Growth enhancement is stimulated by the ionic rather than osmotic effect of NaCl

The ice plants enhanced their growth with 50–200 mM NaCl (Figure 1(a)), which are similar concentrations to those reported as optimal for the ice plants grown hydroponically (Flowers et al., 1977). These results indicate that the halophilism of this halophyte occurs at high NaCl levels as observed in other halophytes (Flowers et al., 1977), such as *Sesuvium portulacastrum* (Wang et al., 2012) and *Salicornia europaea* (Lv et al., 2012). The growth of the suspension-cultured cells was also promoted by NaCl at the concentrations as observed in the whole plant except at 200 mM (Figure 1). These results suggest that the suspension-cultured cells are usable as a model system in the studies on the mechanisms of halophilism. The maximum growth of the cells was obtained with 100 mM NaCl (Figure 1(b–d)). Under this salinity level, the number of cells (Figure 3(a)) and cell size (Figure 3(b)) increased. The growth of cells was enhanced significantly in the medium containing NaCl more than that with PEG. Osmotic pressure has some positive effects on the cell growth, but the degree of growth enhancement was lower than that in the NaCl treatment under same osmotic conditions (Figure 2(c,d)). This was consistent with the results reported by Vera-Estrella et al. (1999).

High salinity increase the intercellular accumulations of ions, which disturbs ion homeostasis and osmotic pressure that would be attributed to the reduction of plant growth (Flowers & Colmer, 2008). However, the growth enhancement by salinity (Figure 1) suggests that the ice plant could efficiently regulate and use the ions positively for the promotion of the cell elongation and cell division (Figure 3). Halophyte that grows under salinity uptake Na^+ and Cl^- to increase osmotic pressure inside cell and water is incorporated into cells along with gradient of osmotic pressure across the

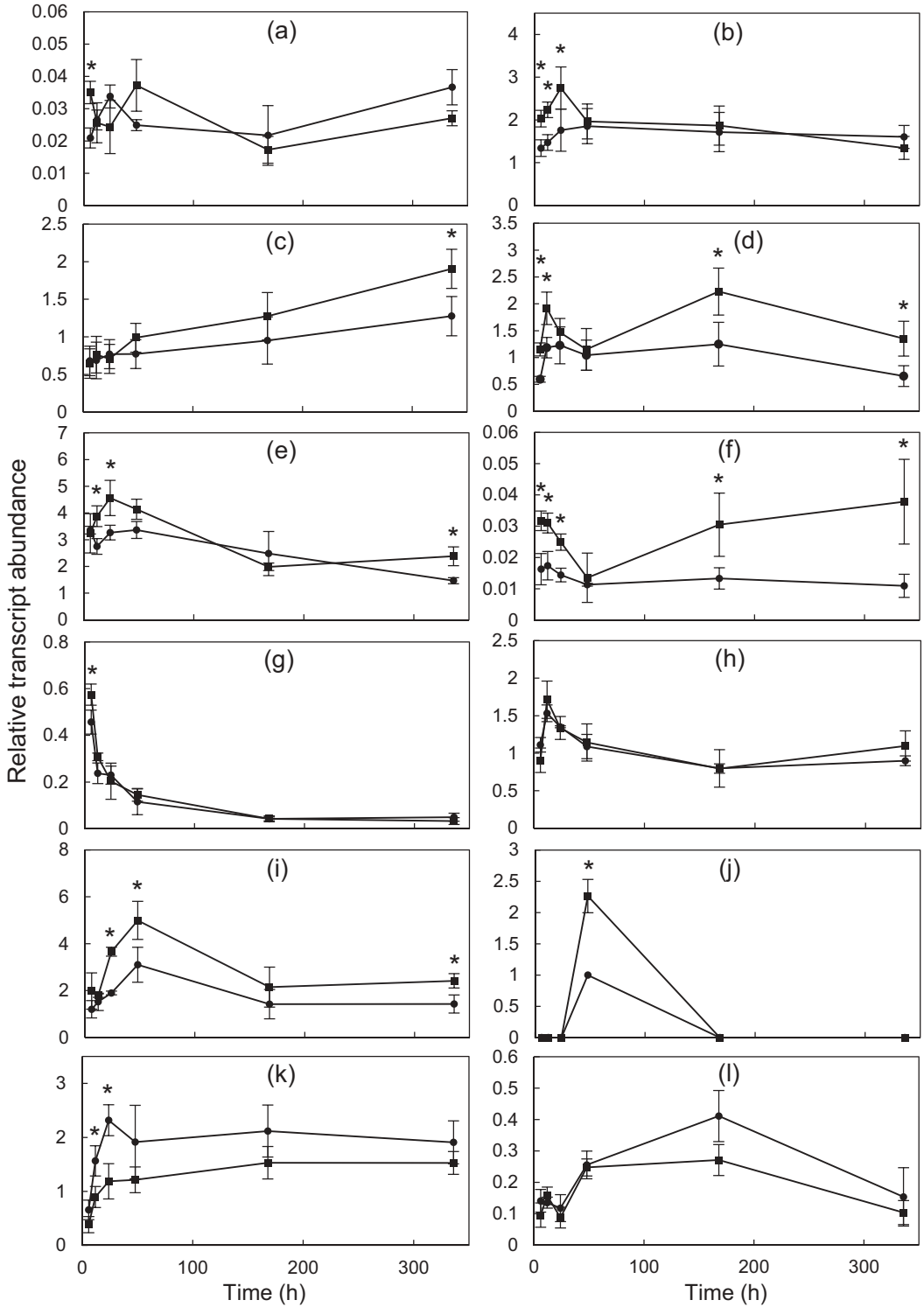

Figure 6. Relative transcript abundance of ion homeostasis-related genes in the cells.

membrane, and cell wall elasticity increase and cell elongate due to increased turgor pressure, which generated by incorporation of water. Improving cell water status is required to promote cell growth (elongation and division of cells). To maintain the levels of Na^+ and Cl^- in cytosol properly, Na^+ and Cl^- are sequestrated into vacuole (using chemical gradient of H^+ which generated by H^+-ATPse). As decrease of osmotic potential in vacuole the compatible solutes are synthesized in cytosol for osmotic adjustment. These sequential reactions have been reported as crucial mechanism responsible for maintenance of cell growth under salinity

(Flowers & Colmer, 2008; Flowers et al., 1977; Shabala, 2013) and the mechanism would also be required for the halophilism.

The enhanced accumulation of Na^+, Cl^-, K^+, and NO_3^- associated with the halophilism

The contents of PO_4^-, SO_4^{2-}, NH_4^+, Mg^{2+}, and Ca^{2+} did not significantly change, except for a slight decrease of PO_4^-, in the suspension-cultured cells treated 50–100 mM NaCl (Figure 4(a)). Whereas, the accumulation of Na^+ and Cl^- significantly increased (Figure 4(b)) and positively correlated with the growth (Figure 5(a,b)), indicating that the enhanced salt accumulation is required for the growth enhancement. This phenomenon has also been found in several halophytes, such as *Salicornia bigelovii* Torr. (Yamada et al., 2016), *S. europaea* (Lv et al., 2012), *S. portulacastrum* (Wang et al., 2012), and *Suaeda salsa* (L.) Pall (Mori et al., 2006). High accumulation of Na^+ and Cl^- is considered to act as an effective osmotic adjuster to maintain or increase turgor pressure in halophytes under salinity (Kaburagi et al., 2014; Lv et al., 2012; Shabala, 2013). The increase of cell size (Figure 3(b)) suggests that Na^+ and Cl^- have a role for increasing turgor pressure, which induces the growth enhancement of the ice plant under favorable NaCl conditions.

K^+ and NO_3^- are essential nutrients for plants, and their accumulation in cells would affect plant growth (Adams & Shin, 2014; Kaburagi et al., 2014). The K^+ and NO_3^- uptake in glycophytes grown under saline conditions are limited by antagonistic inhibitions of Na^+ and Cl^-, respectively, which cause growth reduction due to the K^+ and NO_3^- deficiency (Adams & Shin, 2014). In contrast, many halophytes are able to maintain the K^+ and NO_3^- uptake for their survival under salt stress (Flowers & Colmer, 2008; Shabala, 2013). In the present study, the contents of K^+ and NO_3^- were also maintained or decreased in the cells under salt stress conditions of 200–400 mM NaCl (Figure 4(b)), but they significantly increased under favorable NaCl conditions of 50–100 mM NaCl (Figure 4(b)). The contents of K^+ and NO_3^- also showed positive correlations with the growth (Figure 5(c,d)). These results suggest that the accumulation of K^+ and NO_3^- plays important roles in the halophilism by the promotion of activity for K^+ and NO_3^- requiring physiological processes. However, the K^+ and NO_3^- accumulation only increased when the Na^+ and Cl^- accumulation increased (Figure 4(b)), suggesting that NaCl promotes the K^+ and NO_3^- uptake. In salt tolerant wheat and several halophytes, NaCl stimulates K^+ uptake in the plants grown under K^+ limitation in growth medium (Flowers et al., 1977; Krishnasamy, Bell & Ma, 2014). NO_3^- uptake enhanced by NaCl was also observed in several halophytes under optimal salt conditions, such as *S. bigelovii* Torr and *Beta vulgaris* var. (Kaburagi et al., 2014; Yamada et al., 2016). These observations support the idea that the NaCl-induced K^+ and NO_3^- uptake is contributed to the halophilism of the ice plant.

The ion homeostasis-related genes associated with the halophilism

The enhanced accumulation of Na^+, Cl^-, K^+ and NO_3^- (Figure 4(b)) indicates that the cells regulated the homeostasis of these ions which promotes the growth (Figures 1 and 3). As a result, the expression analysis of genes encoding ion homeostasis-related factors would elucidate physiological significances of the ion accumulation and related genes. Halophytes can incorporate Na^+ and Cl^- into cytoplasm as a response to salinity (Flowers & Colmer, 2008; Flowers, Munns & Colmer, 2014). HKT-type transporters are considered to mediate Na^+ and/or K^+ uptake from saline environments (Himabindu et al., 2016). The expression of *McHKT1* is associated with the Na^+ uptake in the ice plants stressed with 400 mM NaCl, which increases transiently in leaves after 6–10 h of salt treatment (Su et al., 2003). In the present study, the increased expression of *McHKT1* in the salt-treated cells was observed at 6 and 48 h after the onset of treatment (Figure 6(a)), indicating that this gene was also induced for the Na^+ uptake under NaCl conditions that induce the halophilism (Figures 4(b) and 7). The Cl^- uptake is mediated by CCC transporters which are divided into three groups: K^+/Cl^- cotransporters, Na^+/Cl^- cotransporters, and Na^+-K^+/Cl^- cotransporters (Chen et al., 2016). In rice, OsCCC1 transporter was found to be involved in K^+ and Cl^- incorporation in the plant under salinity (Colmenero-Flores et al., 2007). The increased expression of *McCCC1* in the salt-treated cells (Figure 6(b)) suggests that *McCCC1* has the role for the incorporation of Cl^- and either K^+ or Na^+ into the cells (Figures 4(b) and 7).

Due to the toxicity of excess Na^+ and Cl^- in cytoplasm, they are sequestrated into vacuole depending upon a proton gradient generated by V-ATPase (Shabala, 2013). The Na^+ and Cl^- sequestration into vacuole are mediated by members of the tonoplast CLC and NHX family (Li et al., 2006). In previous reports, the enhanced expression of *McNHX1* and *McVmac1* are also required for the Na^+ sequestration in the ice plants under salt stress conditions (Cosentino et al., 2010; Tsiantis et al., 1996). In soybean, the *GmCLC1* is induced for the Cl^- sequestration in the salt-stressed plants, and its overexpression confers salt tolerance in transgenic

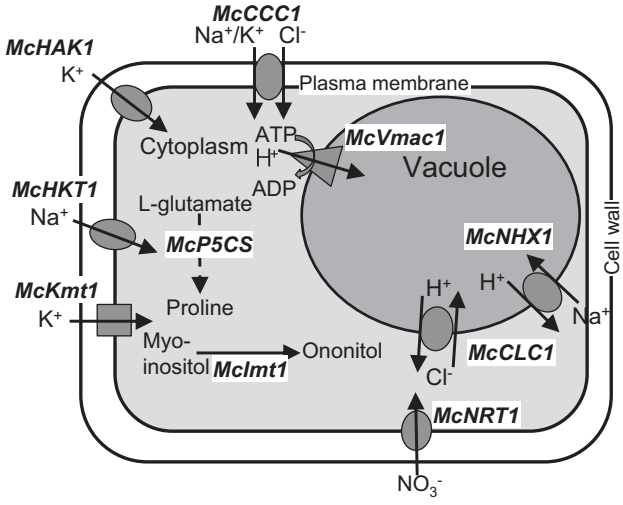

Figure 7. A proposed model including ion homeostasis-related genes, which are as factors involved in uptake of Na^+, Cl^-, K^+, NO_3^-, synthesis of proline and ononitol, water incorporation, and cell wall elasticity in the ice plant.

tobacco and *Arabidopsis* (Li et al., 2006; Wei et al., 2016). In the present study, we observed the increased expression of *McNHX1, McCLC1,* and *McVmac1* in the salt-treated cells (Figure 6(c–e)), suggesting that these genes are also induced for sequestrating Na^+ and Cl^- into the vacuole (Figure 7).

To maintain the homeostasis of K^+ and NO_3^-, plants need to induce the expression of genes for K^+ and NO_3^- transporters and/or channels (Adams & Shin, 2014; Himabindu et al., 2016). The expression of HKT-type, HAK/KUP-type transporters, and Shaker-type channels are considered to be enhanced for K^+ homeostasis in halophytes in response to salt stress (Flowers & Colmer, 2008; Himabindu et al., 2016). In the ice plant, the expression of *McHAK1* is enhanced to support the K^+ uptake in the salt-stressed plants (Su et al., 2002). However, in the present study, it was not increased in the salt-treated cells (Figure 6(h)). It is likely that *McHAK1* is only induced for facilitating K^+ uptake when the uptake through other K^+ transport systems, particularly K^+ channels, is limited by high salinity that causes K^+ starvation of the cells (Su et al., 2002). Different from *McHAK1*, the expression of *McKmt1* was enhanced in the salt-treated cells during the first 12 h after the onset of treatment (Figure 6(g)). These results indicate that the enhancement of K^+ uptake is required in the salt-treated cells (Figure 4(b)) and *McKmt1* would contribute to the K^+ uptake in the cells (Figure 7). In the ice plants stressed with 400 mM NaCl, the expression of *McKmt1* is also induced in a similar pattern (Su et al., 2001). The *McNRT1*, which contributes to NO_3^- uptake for the ion homeostasis in the leaves of salt-stressed ice plants with 400 mM NaCl (Popova et al., 2003), expressed at significantly high levels in the salt-treated cells (Figure 6(f)). This result suggests that *McNRT1* has an important role in the NO_3^- accumulation under saline conditions (Figures 4(b) and 7).

To maintain the osmotic balance between the cytoplasm and vacuole in which Na^+ and Cl^- are sequestrated, the ice plants synthesize ononitol and proline in the cytoplasm for the osmotic adjustment (Agarie et al., 2009; Thomas et al., 1992; Vera-Estrella et al., 1999). The salt-induced gene expression of enzymes catalyzing the synthesis of proline (*McP5CS*) and ononitol (*McImt1*) was also observed in the ice plant (Figure 7) (Oh et al., 2015; Vernon & Bohnert, 1992). In the present study, the *McP5CS* expressed at significantly high levels in the salt-treated cells (Figure 6(i)), and the higher transcript abundance of *McImt1* also observed in the salt-treated cells at 48 h after the onset of treatment (Figure 6(j)). These results suggest that the increased osmotic pressure in the cytoplasm would be required in the cells in the presence of NaCl for inducing the halophilism.

The osmotic adjustment in the salt-treated cells promoted water incorporation. The water influx increased turgor pressure and cell wall elasticity, which induced the cell elongation (Figure 3(b)). At least nine members in *Mip* gene family related to water incorporation have been identified in the ice plant, and their expression is distinct and associated with recovery of leaf turgor in the plants under salt stress (Kirch et al., 2000; Yamada et al., 1995). For example, the expression of *McMipB* is not noticeably affected while that of *McMipA* and *McMipC* are increased (Yamada et al., 1995). However, in the present study, the expression of *McMipC* did not increase in the salt-treated cells (Figure 6(k)). There might have a different regulatory mechanism in the expression of the *Mip* genes for water incorporation in the ice plants under favorable NaCl conditions. The cell wall elasticity in plants is determined by cell wall-modifying enzymes, such as expansins, XTHs, and β-1,4-glucanases, but the roles of these enzymes in salt tolerance are still unclear (Cosgrove, 2005; Han et al., 2013). In salt tolerant *P. euphratica* species, the increased expression of *PeXTH* was found in salt-induced succulent leaves that are formed by the increase of cell number and cell size (Han et al., 2013; Ottow et al., 2005). In the present study, however, the expression of *McXTH* that encodes an XTH enzyme did not increase in the salt-treated cells (Figure 6(l)), suggesting that the ice plant might have a different regulatory mechanism of the expression of *McXTHs* for the cell wall elasticity under salinity. In summary, the results of expression analysis indicate that *McHKT1, McCCC1, McCLC1, McNHX1, McVmac1, McKmt1, McNRT1, McP5CS*, and *McImt1* might play important roles in the ion homeostasis and osmotic adjustment in the ice plants for the salt-promoted growth (Figure 7).

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