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Differential tolerance to high salt with regard to cell growth and superoxide dismutase (SOD) activity in calluses of the halophyte *Suaeda maritima* from Japan and Egypt

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Abstract

To determine the mechanism of regulation of superoxide dismutase (SOD; EC 1.15.1.1) isozymes in halophytes at the cellular level, we isolated calluses from *Suaeda maritima* plants native to Japan and Egypt. Calluses from Egyptian *S. maritima* could grow in a high salt medium (~400 mM NaCl), whereas Japanese *S. maritima* calluses could not. The expression characteristics of SOD isozyme genes encoding two CuZn-SOD proteins, two Mn-SOD proteins, and one Fe-SOD protein in the two callus lines were analyzed. All SOD isozyme genes expressed in leaves were also expressed in both callus lines grown in normal medium and their expression levels were not affected by high salt. In-gel SOD activity assay after native-polyacrylamide gel electrophoresis revealed that both callus lines possessed a CuZn-SOD and an Fe-SOD as major SOD isozymes in normal medium. When calluses were grown on high salt medium, both SOD isozyme activities were decreased in Japanese calluses, while they were stable or slightly increased in Egyptian calluses. When calluses were incubated in normal medium with excess copper (Cu, ~100 μM), callus growth of both callus lines was less affected. In excess Cu, CuZn-SOD activity was greatly increased in both callus lines, while Fe-SOD activity was decreased in Japanese calluses and was stable in Egyptian calluses. Since both Fe-SOD and CuZn-SOD activities exhibited a positive association with growth tolerance to high salt, they may play a role in salt tolerance in callus cells. In addition, our results indicate that the Fe-SOD activity contributes to copper stress tolerance.

Keywords: *Suaeda maritima*; callus; high salt; salt tolerance; superoxide dismutase.
Abbreviations: 2,4-D_2.4-dichlorophenoxyacetic acid; MDA_malondialdehyde; MS_Murashige and Skoog; SOD_superoxide dismutase; CuZn-SOD_copper-zinc SOD; Fe-SOD_iron SOD; Mn-SOD_manganese SOD.

Introduction

Salinity is a major and increasing cause of loss of agricultural land and productivity. Halophytes are a valuable source of information about the molecular and physiological mechanism of salt stress tolerance (Bose et al., 2014; Flowers and Colmer, 2008; Song and Wang, 2015). The halophyte *Suaeda maritima* is found along seashore and inland areas worldwide and the physiological properties of European (Flowers, 1972; Flowers and Colmer, 2008; Wang et al., 2007; Wetson et al., 2008) and India (Sahu and Shaw, 2009a, b) varieties have been studies. The array of SOD isozymes that is present is affected by internal and external factors (Bowler et al., 1994; Ozgur et al., 2013). Since *Suaeda* groups exhibit high phenotypic plasticity due to their ability to change their vegetative characteristics in response to external factors, including salinity (Schutze et al., 2003), physiological characterization of *S. maritima* from regions other than Europe or India would also be valuable. The production of reactive oxygen species (ROS) increases in plants under stress conditions, such as salt stress. SOD catalyzes the dismutation reaction of the superoxide radical ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen and plays an important role in protecting cells from oxidative damage caused by ROS (Bowler et al., 1992). In plants, SOD enzymes are classified into three isotypes based on their metal cofactors, namely, CuZn-SOD, Fe-SOD, and Mn-SOD (Bowler et al., 1994). CuZn-SOD is localized to the cytosol and chloroplast, Mn-SOD is located in the mitochondria and peroxisomes and Fe-SOD is localized predominantly to the chloroplast. Previous studies identified in-gel SOD activity bands from extracts of *S. maritima* (Mallik et al., 2011) and *S. salsa* (Wang et al., 2004; Zhang et al., 2005). However, the band pattern of SOD isozymes varied in different studies. To determine the factor inducing phenotypic variation in SOD isozyme compositions in *Suaeda*, we recently analyzed the expression of SOD isozymes using the aseptic *S. maritima* from Japan and Egypt (Mohamed et al., 2015). Both materials differed in genetic distance based on ITS sequences, but showed the same SOD isozyme composition (Mohamed et al., 2015). In-gel SOD activity assay revealed that at least three CuZn-SOD activity bands, and one Fe-SOD band, are present in the leaves of both varieties. One of the important features of the SOD isozymes from leaves of Japanese and Egyptian *S. maritima* is that Fe-SOD is the major SOD isozyme, as no or minor Fe-SOD activity was present in prior reports on *Suaeda* (Mallik et al., 2011; Wang

et al., 2004; Zhang et al., 2005). Since the Fe-SOD gene was not isolated from *Suaeda* species yet, we isolated a Fe-SOD gene from both sources (Mohamed et al., 2015). The deduced amino acid sequences of Fe-SOD suggest that Fe-SOD localizes to the chloroplast. To determine the mechanism of salt tolerance, callus cells were produced from halophytes (Bajji et al., 1998; Smith and McComb, 1981; Sharma and Ramawat, 2014). The first study of growth characterization of calluses from halophytes was performed using *S. maritima* collected from the coast of Germany and showed that callus growth was promoted in lower salt concentrations (~100 mM NaCl), but was inhibited considerably in higher salt concentration (~400 mM) (Hedenström and Breckle, 1974). Calluses from *S. nudiflora* (Cherian and Reddy, 2003) and *S. australis* (Smith and McComb, 1981) did not exhibit growth under conditions of lower salt levels and high salt concentrations. Tanimoto et al. (1997) reported that a green callus of *S. japonic*a could grow in high salt medium, but not a yellowish callus. They speculated that chloroplast development is involved in high-salt tolerance. Although green *S. japonica* calluses are valuable for studying the salt tolerance mechanism at cellular level, further characterization is difficult, because green calluses are generally hard to maintain. Therefore, *Suaeda* calluses were not previously used for analysis of salt tolerance. In our previous report, *S. maritima* plants from Japan and Egypt were found to possess the same SOD isozyme compositions under aseptic conditions, but had different response to high salt treatment (Mohamed et al., 2015). The most prominent difference is that Egyptian *S. maritima* plants maintained elevated levels of SOD activity even under low stress condition, while Japanese *S. maritima* had reduced activity level. We expected that differences in the regulation of SOD expression between the two *S. maritima* varieties would be due to their genetic background. To determine the mechanism of regulation of SOD isozymes in *S. maritima* at cellular level, we isolated calluses from *S. maritima* from Japan and Egypt and examined salt tolerance with regard to cell growth and superoxide dismutase activity in both callus lines.

Results and Discussion

Phenotypic observation of callus tissues during callus establishment

Fig. 1 shows the process for the establishment of calluses. Sterilized seeds were sown on normal MS agar medium without phytohormone and germinates were cultured for 2 weeks (Fig. 1 a, e). Then hypocotyls were cut from seedlings and transferred to MS agar medium containing 2,4-D and kinetin. Yellowish calluses were induced from both ends of the cut hypocotyls (Fig. 1 b, f). Callus tissues were initially maintained in the dark for 4 months (Fig. 1 c, g), then transferred to light conditions. Callus masses were maintained at 3 weeks intervals on normal MS agar medium for 6 months (Fig.1 d, h). Japanese *S. maritima* calluses were yellowish throughout establishment. In contrast, Egyptian *S. maritima*, hypocotyls were reddish (Fig. 1e) and calluses sometimes produced brownish cells during subcultures in the dark (Fig. 1g). However, established calluses in the light were yellowish (Fig. 1h).

Effect of high salt on callus growth

To determine the effect of salt concentration on callus growth, calluses grown in MS medium with phytohormone were transferred to the same MS medium with different concentrations of NaCl and incubated for 15 days (Fig. 2 a). Before and after incubation, the fresh callus weight of callus was measured. In medium without NaCl, the relative fresh weight growth of Japanese *S. maritima* calluses was similar to that of Egyptian calluses. However, the growth of Japanese calluses was inhibited in 100 mM NaCl and the inhibition increased with increasing salt concentration. In contrast, the growth of Egyptian calluses was increased at 100 mM or 200 mM NaCl. To examine whether the growth rates of the two callus types were stable, callus growth was analyzed at the second and third transfer cycles (Fig. 2b). Japanese calluses did not grow in 200 mM NaCl or 400 mM on the second transfer (Fig. 2b, Supplementary Fig. 2 b and c), while Egyptian calluses continued to grow in 200 mM and 400 mM NaCl on the second and third transfer (Fig. 2b); moreover, Egyptian calluses could grow in high salt medium on the fifth transfer (Supplementary Fig. 2 e and f). Egyptian calluses grew in 400 mM NaCl for two weeks, although they became brownish; their growth ceased thereafter (Supplementary Fig. 2 i). On the other hand, Egyptian calluses in 200 mM grew for more than 30 days without transfer to new medium (Supplementary Fig. 2 h). These findings suggest that Egyptian calluses possess high salt tolerance, while Japanese calluses are sensitive to high salt.

Expression of genes encoding SOD isozymes in response to high levels of salt

RT-PCR analysis showed that all SOD isozyme-encoding genes expressed in leaves (Mohamed et al., 2015) were also expressed in calluses (Fig. 3). The expression levels of the SOD genes in calluses were unaffected by high salt, except that the expressions of the extracellular CuZn-SOD gene in Japanese *S. maritima* was low in the no salt medium, but was increased in the high salt medium. Responses in the transcript levels of genes encoding choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), which are involved in glycine betaine biosynthesis, differed between Japanese and Egyptian calluses. In the absence of salt, *CMO* and *BADH* transcripts were not detected in Japanese callus, but were detected in Egyptian calluses. In 400 mM NaCl, transcripts were diminished in Japanese calluses, but maintained at high levels in Egyptian calluses. High salt tolerance, and high *CMO* and *BADH* gene expression, were also observed in Egyptian *S. maritima* leaves (Mohamed et al., 2015).

In-gel SOD activity

To address the involvement of SOD isozymes in the salt tolerance of Japanese and Egyptian calluses, in-gel SOD activity staining was performed. SOD isozymes in leaves and roots were also analyzed as control (Fig. 4). SOD isozymes were differentiated based on their response to inhibitors (Hernández et al., 1999). CuZn-SOD is inhibited by KCN and H_2O_2, Fe-SOD is inhibited by H_2O_2 only and Mn-SOD is resistant to both KCN and H_2O_2. In callus cells grown in medium without NaCl, CuZn-SOD and Fe-SOD were the major SOD isozymes, and were detected in leaves, roots (Fig. 4) and stems (Mohamed et al., 2015). On the other hand, Mn-SOD activity was observed in roots in Egyptian calluses (Fig. 4) and stems in Japanese and Egyptian *S. maritima* plants (Mohamed et al., 2015), but not in both callus lines. These findings indicate that callus cells possess a simple composition of CuZn-SOD and or Fe-SOD when grown under no salt conditions. To examine the response of SOD isozyme activity to high salt, callus cells were incubated in

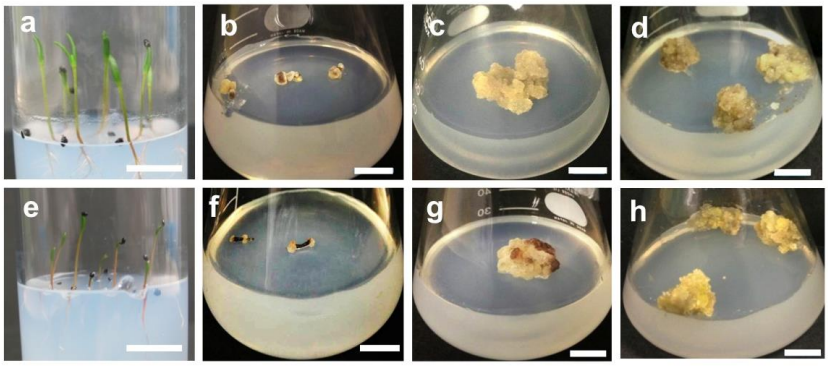

Fig 1. Isolation of calluses from *Suaeda maritima* from Japan (a-d) and Egypt (e-f). (a, e) Seedlings were grown on MS agar medium without phytohormone. (b, f) Excised hypocotyls were incubated on MS agar medium with 1 µM 2,4-D and 1 µM kinetin in the dark. (c, g) Callus masses produced at the edge of hypocotyl were transferred to new medium and subcultured in the dark at monthly interval. (d, h) Dark grown calluses were transferred to the light and established calluses were subcultured at three weeks interval for 6 months. Bar 1 cm.

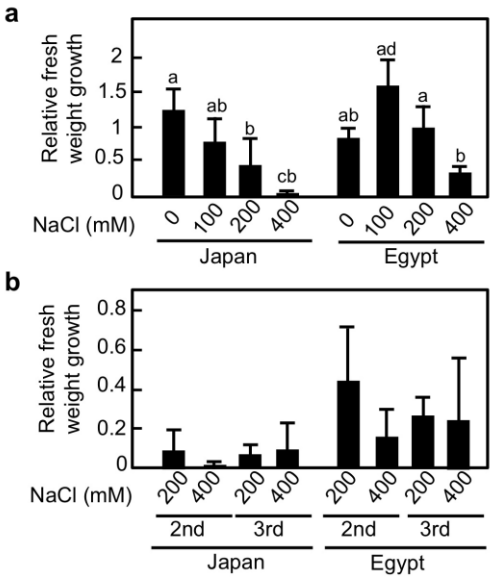

Fig 2. Callus growth on the first passage (a) and following two passages (b) through high salt medium. (a) Japanese and Egyptian *S. maritima* calluses grown on normal MS agar medium were explanted on normal MS medium with different concentrations of NaCl and incubated for 15 days. RFWG = [(W1-W0)]/W0, where W0 and W1 are the initial fresh weights of the callus before and after 15 days. (b) Calluses after incubation on the first passage were explanted to fresh medium with the corresponding salt concentrations and incubated. After incubation of calluses on the second (15 days) and third (15 days) passages, RFWG was measured. Values are means ± SD (n = 3). Means denoted by the same letter were not significantly different at $P < 0.05$ according to one-way ANOVA and the LSD test. Since mean values in Egyptian calluses on the second and third passages showed high SD values, statistical analysis was not performed in (b).

different salt concentrations and cell-free extracts were subjected to in-gel SOD assay (Fig. 5a). In Japanese calluses, Fe-SOD and CuZn-SODI activity was increased in 100 mM NaCl but decreased in higher salt concentration. In Egyptian calluses, a new CuZn-SOD IV band was induced by 100 mM NaCl and Fe-SOD and CuZn-SOD I activity was stable or slightly increased in high salt. Callus growth and SOD activities showed the same response to high salt, although their responses differed between Japanese and Egyptian calluses (Figs. 2 and 5a). Therefore, it is expected that differential effects of high salt on the two calluses are due to ROS generation. To address this possibility, the effect of high salt on MDA generation in Japanese and Egyptian calluses was analyzed (Fig. 5b). The profile of MDA content under high salt conditions was similar to that for cell growth and SOD activity in Japanese and Egyptian calluses.

Effect of copper on growth and SOD isozyme expression

To examine the effect of copper on the oxidative stress responses in calluses, callus growth and MDA content in calluses grown in excess Cu medium were measured (Fig. 6). In Japanese calluses, excess Cu slightly decreased callus growth but increased MDA content. In contrast, excess Cu did not affect the growth of Egyptian calluses and MDA accumulation. Next, the transcript levels of SOD isozyme genes and glycine betaine biosynthetic genes were analyzed in Japanese and Egyptian calluses at different Cu concentrations

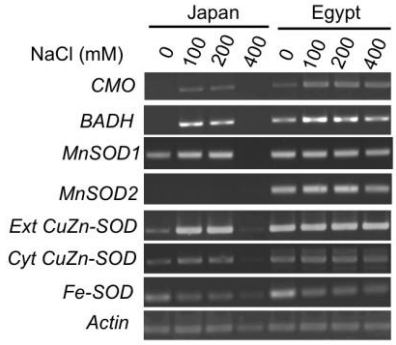

Fig 3. Transcript levels of SOD isozyme genes in *S. maritima* calluses grown under unstressed and high-salt stress conditions. Calluses grown on normal medium were transferred to medium containing different concentrations of NaCl and incubated for 15 days. Total RNA was extracted from calluses grown on medium with or without NaCl. RT-PCR was performed.

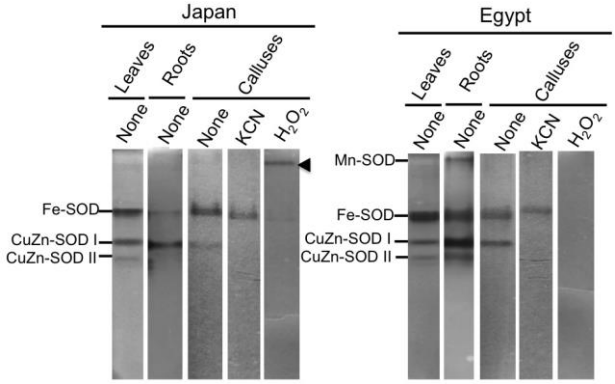

Fig 4. Comparison of SOD isozyme compositions in leaves, roots and calluses. Crude extracts was prepared from the leaves and roots of 7-cm-tall *S. maritima* plants grown on medium without NaCl under aseptic conditions. Crude extracts were also prepared from calluses grown for 7 days on normal medium without NaCl. The extracts (20 µg protein for Japanese samples, 30 µg protein for Egyptian samples) were electrophoresed on a native polyacrylamide gel. After electrophoresis, gels with the extracts from leaves, roots and calluses were subjected to SOD activity staining for 30 min. The remaining gels with callus extracts were treated with 3 mM KCN or 10 mM H_2O_2 for 1 hr. After treatment, the gels were exposed to staining solution for 30 min. The arrowhead indicates the catalase activity band.

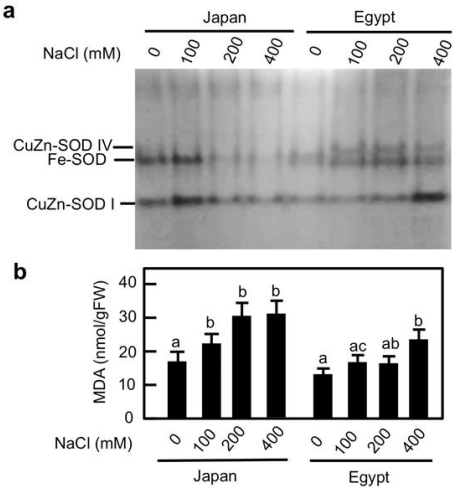

Fig 5 Effect of high salt on SOD isozyme activity in Japanese and Egyptian *S. maritima* calluses. Calluses grown in normal medium were transferred to medium containing different concentrations of NaCl and cultured for 15 days. Crude extracts were prepared from calluses. (a) The extracts (60 µg protein) were electrophoresed on a native polyacrylamide gel and in-gel SOD activity assay was performed. (b) MDA content in calluses. Calluses grown on normal medium were transferred to medium containing different concentrations of NaCl and cultured for 15 days. MDA content was measured as nmole per g fresh weight. Values are means ± SD (n = 3). Means denoted by the same letter were not significantly different at $P < 0.05$ according to one-way ANOVA and the LSD test.

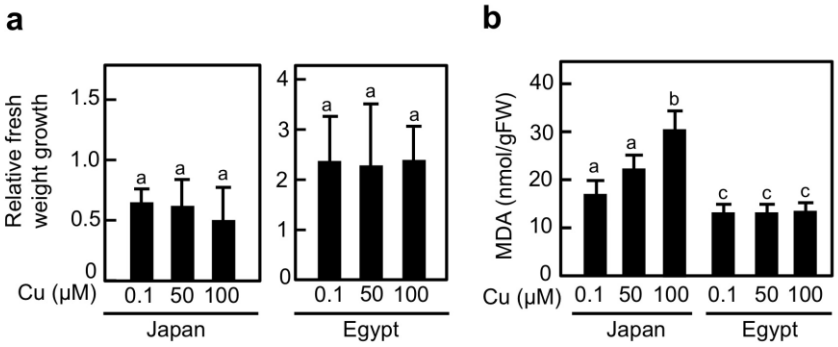

Fig 6. Callus growth and MDA content in excess Cu medium. (a) Calluses grown on normal MS agar medium (0.1 μM Cu) were explanted on normal MS medium with 0.1, 50 or 100 μM Cu and incubated for 10 days. The fresh weights of callus pieces before and after incubation were measured and relative fresh weight growth was calculated. (b) After measurement of callus pieces, calluses were used for MDA measurement. Values are means ± SD (n = 3). Means denoted by the same letter were not significantly different at $P < 0.05$ according to one-way ANOVA and the LSD test.

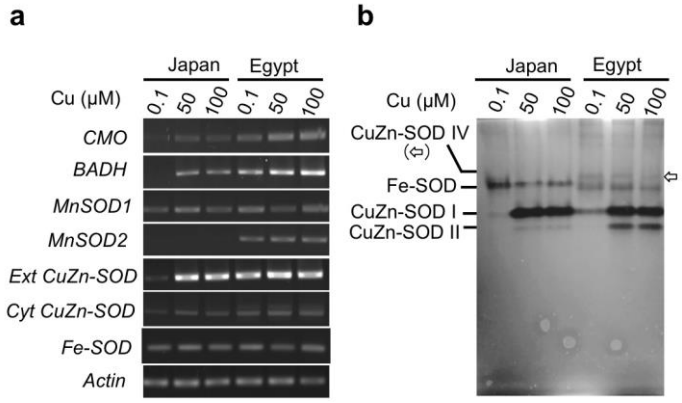

Fig 7. Effect of excess Cu on the transcript abundance of SOD isozyme genes (a) and SOD isozyme activity (b) in Japanese and Egyptian *S. maritima* calluses. Calluses grown on normal medium (0.1 μM Cu) were transferred to medium containing 0.1, 50, or 100 μM Cu and cultured for 10 days. (a) Total RNA was extracted from calluses and RT-PCR was performed. (b) Crude extracts were prepared from calluses. The extracts (60 μg protein) were electrophoresed on a native polyacrylamide gel and in-gel SOD activity assay was performed.

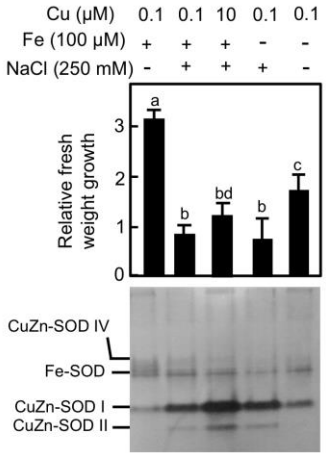

Fig 8. Effect of Fe deficiency and excess Cu on callus growth and SOD isozyme activity in Egyptian *S. maritima* calluses. Calluses grown on normal MS agar medium (0.1 μM Cu and 100 μM Fe) were explanted on MS medium with different concentrations of Cu, Fe and NaCl and incubated for 15 days. The fresh weights of callus pieces before and after incubation were measured and relative fresh weight growth was calculated (n=3). Values are means ± SD (n = 3). Means denoted by the same letter were not significantly different at $P < 0.05$ according to one-way ANOVA and the LSD test. The cell free extracts were prepared from calluses and in-gel SOD activity was analyzed.

(Fig. 7a). In Egyptian calluses, the transcript levels of all genes examined were not affected by excess Cu, while in Japanese calluses, the transcript levels of extracellular *CuZn-SOD*, *CMO* and *BADH* genes were increased by excess Cu. As shown in Fig. 7b, CuZn-SOD I activity was faint in the absence of NaCl in Japanese and Egyptian calluses, but was greatly increased by excess Cu. In contrast, Fe-SOD activity was not affected in Egyptian calluses and decreased in Japanese calluses. CuZn-SOD IV activity was not detected in Japanese calluses even under conditions of excess copper, but was detected in Egyptian calluses; its activity was unchanged by Cu. Since CuZn-SOD IV activity in Egyptian calluses in medium without salt was not detected (Fig. 4), but was detected under conditions of excess Cu (Fig. 7b), its expression appears to be unstable. Therefore, it is unlikely that CuZn-SOD IV plays a principal role in high salt tolerance in Egyptian calluses. Instead, Fe-SOD or CuZn-SOD I may be the main form for salt tolerance. To examine which isozyme is more important for high salt tolerance, effect of Fe deficiency and excess copper on growth of Egyptian calluses in high-salt medium was analyzed (Fig. 8). When the callus was grown in the normal medium depleting Fe and containing 250 mM NaCl, callus growth was reduced to about 30% of the control. Addition of Fe to the same medium did not affect callus growth, while addition of excess Cu slightly promoted the growth. These findings suggest that excess Cu alleviates high-salt stress induced damage. The most prominent feature of Japanese and Egyptian *S. maritima* calluses is that Egyptian *S. maritima* calluses are tolerant to high salt, while Japanese calluses are sensitive to high salt (Fig. 2). When these traits were compared with other *Suaeda* calluses, Japanese *S. maritima* calluses resemble *S. nudiflora* calluses from India (Cherian and Reddy, 2003), *S. australis* calluses from Australia (Smith and McComb, 1981), and yellow *S. japonica* calluses from Japan (Tanimoto et al., 1997), in that their growth was inhibited at all NaCl levels. In contrast, Egyptian *S. maritima* calluses resemble German *S. maritima* calluses (Hedenström and Breckle, 1974) and green Japanese *S. japonica* calluses (Tanimonoto et al., 1997), because their growth was promoted at lower salt level (43–86 mM) and decreased at high salt levels (~400 mM). Differences in salt tolerance among calluses from *Suaeda* varieties may be due to factors other than genetic variation, because salt tolerant and sensitive calluses were generated from the same callus origin in *S. japonica* (Tanimoto et al., 1997). Illumination and cytokinin are important factors inducing and maintaining chlorophyllous calluses (Fitch and Moore, 1990; Chakravarty and Goswami, 1999). Yellowish *S. japonica* calluses were induced and established in the absence of cytokinin and illumination (Tanimonoto et al., 1997). Calluses of *S. nudiflora* (Cherian and Reddy, 2003) and *S. australis* (Smith and McComb, 1981) were also grown in the dark, although 2,4-D and kinetin were present in the medium. Therefore, low tolerance to high salt in these calluses may be due to inappropriate culture conditions. In contrast, German *S. maritima* calluses (Hedenström and Breckle, 1974) and green *S. japonica* calluses (Tanimonoto et al., 1997) were grown in medium with IAA and kinetin in the presence of light. Based on these examples, it is possible that different culture conditions may produce calluses with different salt tolerances. In this study, cytokinin and illumination were present throughout callus establishment, but both salt sensitive and tolerant calluses were produced. During establishment of *S. maritima* calluses, Japanese *S. maritima* calluses did not produce pigmented cells. In contrast, during the early stages of establishment of stable calluses, pale green calluses were sometimes generated from yellow Egyptian *S. maritima* calluses, but they disappeared in the absence of selection. This suggested that Egyptian yellow calluses might produce green cells by changes in the culture conditions. Since Egyptian yellow calluses showed the same salt tolerance as green *S. japonica* calluses, it is expected that green Egyptian *S. maritima* calluses may have a much higher salt tolerance. A second important feature of *S. maritima* calluses is the close correlation of Fe-SOD and CuZn-SOD I activity to salt tolerance. Fe-SOD and CuZn-SOD I are major SOD isozymes in Japanese and Egyptian *S. maritima* calluses (Fig. 4), but their responses to high salt differed between the two callus lines. Fe-SOD and CuZn-SOD I activities in Japanese calluses were strongly inhibited by 200 mM NaCl, while their levels in Egyptian calluses were less affected in high salt (Fig. 5). In Egyptian calluses, Fe-SOD, CuZn-SOD I, and CuZn-SOD IV activities were detected under high salt. Decreases in total SOD activity were also observed in NaCl-treated *S. nudiflora*, callus cultures, based on the strong inhibition of callus growth in high salt (Cherian and Reddy, 2003). In contrast to calluses, whole *S. nudiflora* plants showed increased SOD activity under salt stress (Cherian and Reddy, 2003), similar to Japanese *S. maritima* plants (Mohamed et al., 2015). Since SOD isozymes in *Suaeda* calluses have not been reported, responses of SOD isozymes in two callus lines were compared. Although CuZn-SOD IV activity in Egyptian calluses was induced by high salt (Fig. 5), it was sometimes produced in the control medium (Fig. 8, Supplementary Fig. 2). Therefore, its contribution to high salt tolerance remains unclear. Since Fe-SOD and CuZn-SOD I activity was positively related to growth tolerance to high salt (Fig. 5), it is possible that they play a role in salt tolerance in callus cells. In previous reports on *Suaeda* (Mallik et al., 2011; Wang et al., 2004; Zhang et al., 2005), no or minor Fe-SOD activity was detected in leaves. In contras, we observed high Fe-SOD activities in the leaves of Japanese and Egyptian plants (Mohamed et al., 2015). We explained that the difference in expression level of Fe-SOD in our system and other studies are due to different culture conditions. However, Fe-SOD activity is present in both chlorophyllous tissues of leaves and stems (Mohamed et al., 2015) and non-chlorophyllous roots and calluses (Fig. 4), indicating that Fe-SOD may play an important role in Japanese and Egyptian *S. maritima* calluses. In addition to Fe-SOD, CuZn-SOD I seems to contribute for high salt tolerance in Egyptian calluses (Fig. 8). In leaves of Egyptian *S. maritima* plants, CuZn-SOD I activity was constitutively at high level (Mohamed et al., 2015). In contrast, in Egyptian calluses, CuZn-SOD I activity was increased by high salt, and excess Cu alleviated the damage of high salt for callus growth. These findings suggest that molecular mechanism in salt tolerance by Fe-SOD is different from that by CuZn-SOD I.

A third prominent feature of Japanese and Egyptian *S. maritima* calluses is differential regulation of Fe-SOD expression. In Japanese *S. maritima* calluses, Fe-SOD activity was decreased by excess Cu, while the transcript level of *Fe-SOD* was not affected by Cu (Fig. 7). In Egyptian calluses, Cu did not affect both Fe-SOD activity and transcript level of Fe-SOD. These results suggest that Fe-SOD activity in Japanese *S. maritima* calluses is regulated at translational or post-translational levels. The same regulation was observed in leaves of Japanese *S. maritima* plants, in which Fe-SOD activity was low under no salt conditions and increased by high-salt treatment, although the transcript level was not affected (Mohamed et al., 2015). Therefore, it is proposed that the translational or post-translational regulation of Fe-SOD is a common property of the intact plants and calluses of Japanese *S. maritima*, while upregulation of Fe-

SOD operates in intact plants and calluses of Egyptian *S. maritima*.

Halophytes are known to accumulate large amounts of metals in their aerial and belowground organs (Lutts and Lefèvre, 2015; Duarte et al., 2013). Milić et al. (2012) reported that *S. maritima* accumulated Cu in aboveground organs, the concentration of which exceeded that in the soil, and suggested that *S. maritima* is valuable for phytoremediation although it is not a hyper-accumulator. Japanese and Egyptian *S. maritima* calluses had relatively high tolerance to excess Cu (Fig. 6a), although Japanese calluses were more sensitive than Egyptian calluses based on MDA accumulation (Fig. 6b). Since CuZn-SOD I is present in all tissues, roots, leaves (Fig. 4), and stems (Mohamed et al., 2015), it is expected to play an important role for protection excess Cu in Japanese and Egyptian *S. maritima* plants. Glycine betaine is accumulated in chloroplasts to protect against salt toxicity or in the cytosol to maintain intracellular osmotic balance between the cytoplasm and the NaCl in the vacuole (Genard et al., 1991; Chen and Murata, 2008; Giri, 2011). Glycine betaine was not accumulated in yellow *S. Japonica* calluses, but was abundant in green calluses (Tanimoto et al., 1997). Since glycine betaine accumulation was promoted by NaCl in *S. japonica* calluses, the higher salt tolerance in green *S. japonica* calluses is explained as the abundant accumulation of glycine betaine (Taninomoto et al., 1998). As shown in Figs. 3 and 7, *CMO* and *BADH* gene transcripts were not detected in Japanese *S. maritima* calluses grown in normal medium, but high salt or excess Cu increased their expression. In leaves of Japanese and Egyptian *S. maritima* plants, transcript levels were lower in plants grown in the absence of salt and increased by salt (Mohamed et al., 2015). We explained this result as the transport of sodium ions from roots to leaves, which induced the expression of *CMO* and *BADH*. In Japanese *S. maritima* calluses, excess Cu also promoted *CMO* and *BADH* gene expression (Fig. 7a), suggesting that excess Cu-induced ROS may activate *CMO* and *BADH*. In contrast, Egyptian *S. maritima* calluses had high transcript levels of *CMO* and *BADH* genes (Fig. 7a). These findings suggest that glycine betaine biosynthetic genes are differentially regulated between Japanese and Egyptian calluses. It further suggests that upregulation of *CMO* and *BADH* genes may contribute to high salt tolerance in Egyptian *S. maritima* calluses.

Materials and Methods

Plant materials and aseptic cultures of plants on vermiculites

Mature seeds of *Suaeda maritima* were collected on the Damietta coast of the Mediterranean Sea in Egypt and on the Ariake Sea coast, Kamiamakusa, Kumamoto, Japan. Procedures for culturing aseptic plants in small glass tube (Mohamed et al., 2015) are briefly described below. The sterilized seeds were germinated and grown to seedlings on MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 1mM MES buffer (pH 5.8) and 0.8% agar in darkness at 4 ˚C for 3 days. The flasks were then transferred to a culture room at 23 ˚C. Germinates were cultured for 7 days under a light intensity of 4 μmol m^{-2} s^{-1} followed by culturing for one month under a light intensity of 30 μmol m^{-2} s^{-1}. The plants were transferred to sterile vermiculite in tube flasks. They were irrigated daily with 20% MS solution without sugar containing 40 μM $FeSO_4$, 40 μM Na-EDTA and 500-μM MES buffer (pH 5.8).

Establishment of callus cultures and callus growth

To isolate calluses, hypocotyls were excised from aseptic seedlings grown on MS medium containing 3% sucrose, 1mM MES buffer (pH 5.8) and 0.8% agar, transferred to the same MS agar medium supplemented with 1 μM 2,4-D and 1 μM kinetin and incubated in the dark. Yellow or white calluses were produced on the edge of the excised hypocotyl after 1 month. The callus mass was subcultured for 4 months in the dark. Calluses were transferred on the continuous illumination (30 μmol m^{-2} s^{-1}) and subcultured for more than 6 months.

Calluses grown on normal MS medium were divided into pieces (100 mg ~500 mg) and transferred to the same medium with different concentrations of NaCl or $CuSO_4$. In all experiments three callus pieces on the flask per treatment were used. Callus pieces were weighed before transfer to fresh medium (W0). They were weighed again after 2 weeks of culture (W1). Relative fresh weight growth (RFWG) was calculated as (W1-W0)/W0.

Preparation of cell free extracts and in-gel SOD activity staining

Leaves and roots (50 mg ~200 mg fresh weight) or calluses (0.2 g fresh weight) were ground at 4 ˚C in 400 μL 20 mM Tris HCl buffer (pH 7.8) containing 1 mM DTT, 1 mM EDTA, 20 mg polyvinylpolypyrrolidone and 20 mg quartz sand. The crude extract was centrifuged at 14,000 x *g* for 10 min and the supernatant was used as the cell free extract. The protein in the supernatant was quantified spectrophotometerically according to the method of Bradford (1976) with bovine serum albumin used as a standard. Non-reducing gel electrophoresis proceeded on 7.5% (w/v) acrylamide using Laemmli's system (1970) without SDS. After non-denaturing PAGE, the gels were stained for SOD activity according to the method reported previously (Mohamed et al., 2015). To differentiate SOD isozymes, gels were pre-incubated in 200 mM K-phosphate buffer (pH 7.8) containing 1 mM EDTA with 3 mM KCN or 10 mM H_2O_2 for 1 h and then incubated in 0.1 mM riboflavin and 0.24 mM nitro blue tetrazolium for 30 min in the buffer and stained by exposure to fluorescence light. The SOD activity on the gels appears as a negative staining. However, the negative staining has been presented in this manuscript as a positive image for the clarity of presentation.

MDA determination

MDA content was measured using spectrophotometric method. Calluses (0.2 g) were homogenized in 5 ml of 0.25% thiobarbituric acid in 10% trichloroacetic acid. The extract was heated at 95 ˚C for 30 min then quickly cooled on ice. The samples were centrifuged at 10,000 x *g* for 30 min. the absorbance of supernatant was measured at 532 nm and 600 nm. The amount of nonspecific absorbance was corrected by subtracting the absorbance at 600 nm from absorbance at 532 nm. The content was calculated using extinction coefficient 155 $mM^{-1}cm^{-1}$ (Stewart and Bewley, 1980).

RNA isolation and RT-PCR analyses

Total RNA was extracted from calluses using RNeasy Plant Mini Kit (Qiagen, USA). RNA was purified from DNA contamination by DNase kit system (Promega, USA). First strand cDNA was constructed by 0.5 μg of total RNA using oligo (dT) containing adaptor primer kit (Takara primescript

II 1st strand cDNA synthesis kit). Genes (GenBank accession numbers) used to design RT-PCR primers are as follows: *CuZn-SOD1* (JQ061159), *CuZn-SOD2* (JQ234923), *Fe-SOD* (LC027286, LC027287), *Mn-SOD1* (LC027288, LC027289, LC027290), *Mn-SOD2* (LC027291), *CMO* (JX629239), *BADH* (JX629240), and *actin* (FJ587488). The sequences of the primers are summarized in Supplementary Table 1. The PCR conditions consisted of initial denaturation at 94 ˚C for 3 min then 35 cycles as the following 94 ˚C for 30 s (denaturation), 58 ˚C for 30 s (annealing), 72 ˚C for 90 s (extension). In the final step, the product exposed to 72 ˚C for 7 min. PCR products were separated by agarose gel electrophoresis for 30 min at 100 V followed by staining with ethidium bromide for 30 min.

Statistical analysis

For callus growth and MDA, the mean values of three replicates with standard deviation were shown in the figures. Statistical analysis was performed by one-way ANOVA and LSD test.

Conclusion

Egyptian *S. maritima* callus growth showed high tolerance to salt, whereas Japanese *S. maritima* calluses were sensitive to salt stress. All of the SOD isozyme genes were unaffected by high salt concentrations. Both CuZn-SOD and Fe-SOD activities were decreased in Japanese *S. maritima* calluses by high salt treatment, while their activities were stable or increased in Egyptian calluses. Therefore, these enzymes may play a role in salt tolerance in callus cells. Egyptian *S. maritima* calluses showed higher tolerance to excess copper than the Japanese calluses. Our results indicated that Fe-SOD play a role in excess copper stress. Therefore, Japanese and Egyptian *S. maritima* calluses will provide alternative materials for studying the mechanism of salt tolerance in halophytes at the cellular level.

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Conflict of interest

The authors have no conflicts of interest to declare.

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