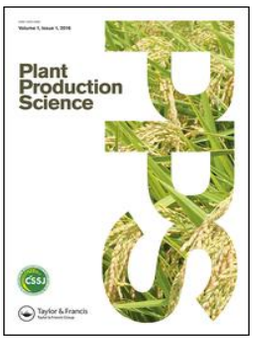

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Effects of Salinity Stress on the Structure of Bundle Sheath and Mesophyll Chloroplasts in NAD-Malic Enzyme and PCK Type C_4 Plants

Eiji Omoto, Mitsutaka Taniguchi and Hiroshi Miyake

(Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan)

Abstract: The effect of NaCl stress on the structure of leaf chloroplasts was investigated in several NAD-Malic enzyme (NAD-ME) and phospho*enol*pyruvate carboxykinase (PCK) type C_4 plant species. Seedlings of the monocot species, except *Zoysia japonica*, grown in 300 mL pots were subjected to salt stress by adding 50 mL of 3% NaCl solution per day to the soil for 5 d after the fourth leaf blades were fully developed. *Z. japonica* and the dicot species, *Amaranthus tricolor*, were also treated with 3% NaCl in a similar manner from 5 wk after germination. Salt stress negatively affected the growth, chlorophyll content and chloroplast structure in all the species. At the ultrastructure level, swelling of thylakoids and disruption of envelopes were more or less observed in mesophyll cell (MC) chloroplasts after salt treatment. The structure of bundle sheath cell (BSC) chloroplasts, on the other hand, was hardly damaged under salt condition although stromal and starch areas were considerably decreased. Furthermore, salinity induced granal development in BSC chloroplasts in most species; the number of thylakoids per granum, granal indices and appressed thylakoid density in salt-treated plants were generally higher than those in control. Since the similar responses have also been reported in all NADP-ME type C_4 species investigated in our previous study, the high sensitivity to salt stress in MC chloroplasts and the granal development in BSC chloroplasts by salinity were considered to be common phenomena in all three C_4 subtypes.

Key words: Bundle sheath chloroplast, C_4 plants, Granal development, Mesophyll chloroplast, NAD-ME type, PCK type, Salinity stress.

Salt accumulation in soil constitutes a severe agricultural problem in many parts of the world, especially in arid and semiarid regions (Zhu, 2001; Borsani et al., 2003). In most saline soil, Na^+ is a major ion that is present in high concentrations (Cumming and Elliot, 1991) and causes the reduction of plant growth and crop yield because of its ion toxicity. Excess salinity induces various detrimental effects on plants such as oxidative stress (Savouré et al., 1999; Zhu, 2001; Yamane et al., 2009), inhibition of photosynthetic activity (Meloni et al., 2003; Shabala et al., 2005) and ultrastructural damage (Rahman et al., 2000; Mitsuya et al., 2000, 2003; Yamane et al., 2003, 2008), and finally arrests plant development and leads to plant death.

C_4 plants are well suited to the growth in high intensity light, arid and warm environments (Black, 1973) and have been considered to have high resistance to salinity stress (Osmond et al., 1982; Stepien and Klobus, 2005). In previous studies conducted from a morphological viewpoint, bundle sheath cell (BSC) chloroplasts of C_4 plants showed higher tolerance to salinity stress than mesophyll cell (MC) chloroplasts, and ultrastructure of BSC chloroplasts remained nearly unaffected by salinity even if MC chloroplasts suffered catastrophic damage (Hasan et al., 2005, 2006; Barhoumi et al., 2007; Omoto et al., 2009). Meanwhile, MC chloroplasts of C_3 plants were also damaged by salinity stress as those of C_4 plants (Salama et al., 1994; Yamane et al., 2003; Fidalgo et al., 2004), in addition, MC and BSC chloroplasts were similarly affected in rice (Rahman et al., 2000).

C_4 plants are divided into three subtypes depending on differences in decarboxylating mechanisms: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and phospho*enol*pyruvate carboxykinase (PCK) types (Hatch, 1999). Each biochemical subtype has particular

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Abbreviations: BSC, bundle sheath cell; GR-RBP, glycine-rich RNA-binding protein; MC, mesophyll cell; PSII, photosystem II; ROS, reactive oxygen species; TEM, transmission electron microscopy; V, vascular bundle.

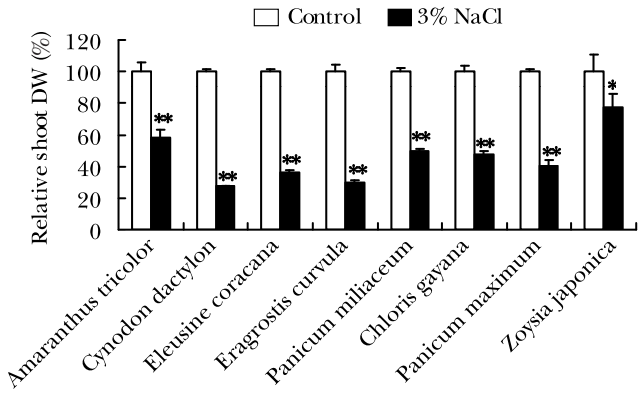

Fig. 1. Effect of 3% NaCl treatment for 5 d on the relative shoot dry weight of NAD-ME and PCK type C_4 plants. Control values were 766.3±47.0 (*Amaranthus tricolor*), 327.9±5.7 (*Cynodon dactylon*), 205.0±3.3 (*Eleusine coracana*), 85.1±3.5 (*Eragrostis curvula*), 251.8±5.1 (*Panicum miliaceum*), 136.7±4.6 (*Chloris gayana*), 320.9±5.1 (*Panicum maximum*), 265.9±29.4 mg (*Zoysia japonica*). Values are means±SE from 7 samples. * and** indicate significant differences from control at $P<0.05$ and $P<0.01$, respectively.

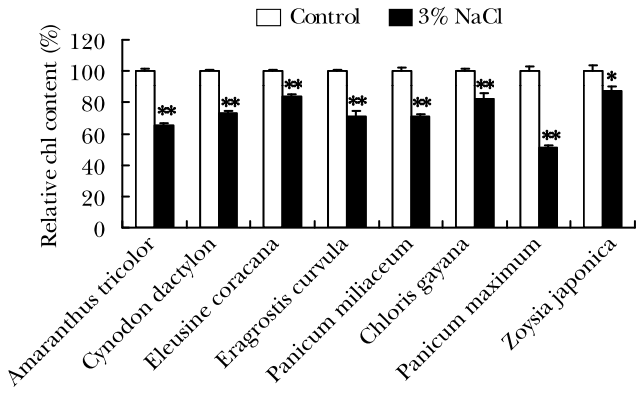

Fig. 2. Effect of 3% NaCl treatment for 5 d on the relative chlorophyll content of NAD-ME and PCK type C_4 plants. Chlorophyll content was measured on a dry weight basis and converted to relative value. Control values were 19.1±0.3 (*Amaranthus tricolor*), 25.4±0.2 (*Cynodon dactylon*), 24.3±0.3 (*Eleusine coracana*), 26.7±0.2 (*Eragrostis curvula*), 29.9±0.7 (*Panicum miliaceum*), 30.5±0.6 (*Chloris gayana*), 31.1±0.9 (*Panicum maximum*), 22.0±0.8 mg g^{-1} (*Zoysia japonica*). Values are means±SE from 7 samples.* and** indicate significant differences from control at $P<0.05$ and $P<0.01$, respectively.

characteristics in leaf anatomies and organelles (Hattersley and Watson, 1992; Dengler and Nelson, 1999; Yoshimura et al., 2004). For example, BSC chloroplasts of NADP-ME type species possess greatly reduced grana (Ghirardi and Melis, 1983), whereas those of NAD-ME and PCK type species contain well-developed grana as MC chloroplasts (Hattersley and Watson, 1992).

In previous studies, BSC chloroplasts of NADP-ME type C_4 species were little damaged by salinity compared with MC chloroplasts, but an interesting ultrastructural change was observed. Hasan et al. (2005, 2006) and Omoto et al. (2009) demonstrated that salinity induced significant granal development in BSC chloroplasts. However, almost no work has focused on NAD-ME and PCK type C_4 species and examined how salinity affects their chloroplast structure.

Based on our previous study (Omoto et al., 2009), we investigated the effect of salinity stress on the chloroplast ultrastructure in NAD-ME and PCK type species in this paper. The aim of this study is to examine the effect of salinity on the granal structure of BSC chloroplasts which have inherently well-developed grana, and the difference in salt sensitivity between BSC and MC chloroplasts.

Materials and Methods

1. Plant materials

Five species belonging to NAD-ME type and three species belonging to PCK type were used; *Amaranthus tricolor* L., *Cynodon dactylon* (L.) PERS, *Eleusine coracana* (L.) Gaertn, *Eragrostis curvula* (Schrad.) Nees and *Panicum miliaceum* L. are NAD-ME type, and *Chloris gayana* Kunth, *Panicum maximum* Jacq and *Zoysia japonica* Steud are PCK type species. Except *A. tricolor* which is a dicot, all species are Poaceae (monocots). These plants were grown in 300 mL plastic cups containing soil and irrigated with tap water in a growth chamber. The growth chamber was controlled at 30/25°C (light/dark), relative humidity of 70%, a 12 hr photoperiod and light intensity of 600 μmol m^{-2} s^{-1}. In the monocot species except *Z. japonica*, seedlings were subjected to salt stress by adding 50 mL of 3% NaCl solution per day to the soil for 5 d after the fourth leaf blades had fully developed. *Z. japonica* and the dicot species, *A. tricolor*, were also treated with 3% NaCl in a similar manner from 5 wk after germination. Control plants were daily watered with tap water.

2. Measurement of growth and chlorophyll content

Growth was analyzed by measuring the dry weight of shoots. In all species except *Z. japonica* and *A. tricolor*, the middle parts of fourth leaf blades were used for measurement of chlorophyll content. *Z. japonica* and *A. tricolor* were also measured using the middle part of the fully developed uppermost leaves. Chlorophyll content was assayed according to Kundson et al. (1977).

3. Transmission electron microscopy (TEM)

Electron microscopic studies were made using the same part of the leaves as that for chlorophyll content analysis. Small segments (about 1 mm×2 mm) of the leaf tissues were prepared, dehydrated with acetone and propylene oxide, and embedded in Spurr's resin as described previously (Omoto et al., 2009). The ultrathin sections were stained with uranyl acetate and lead citrate and

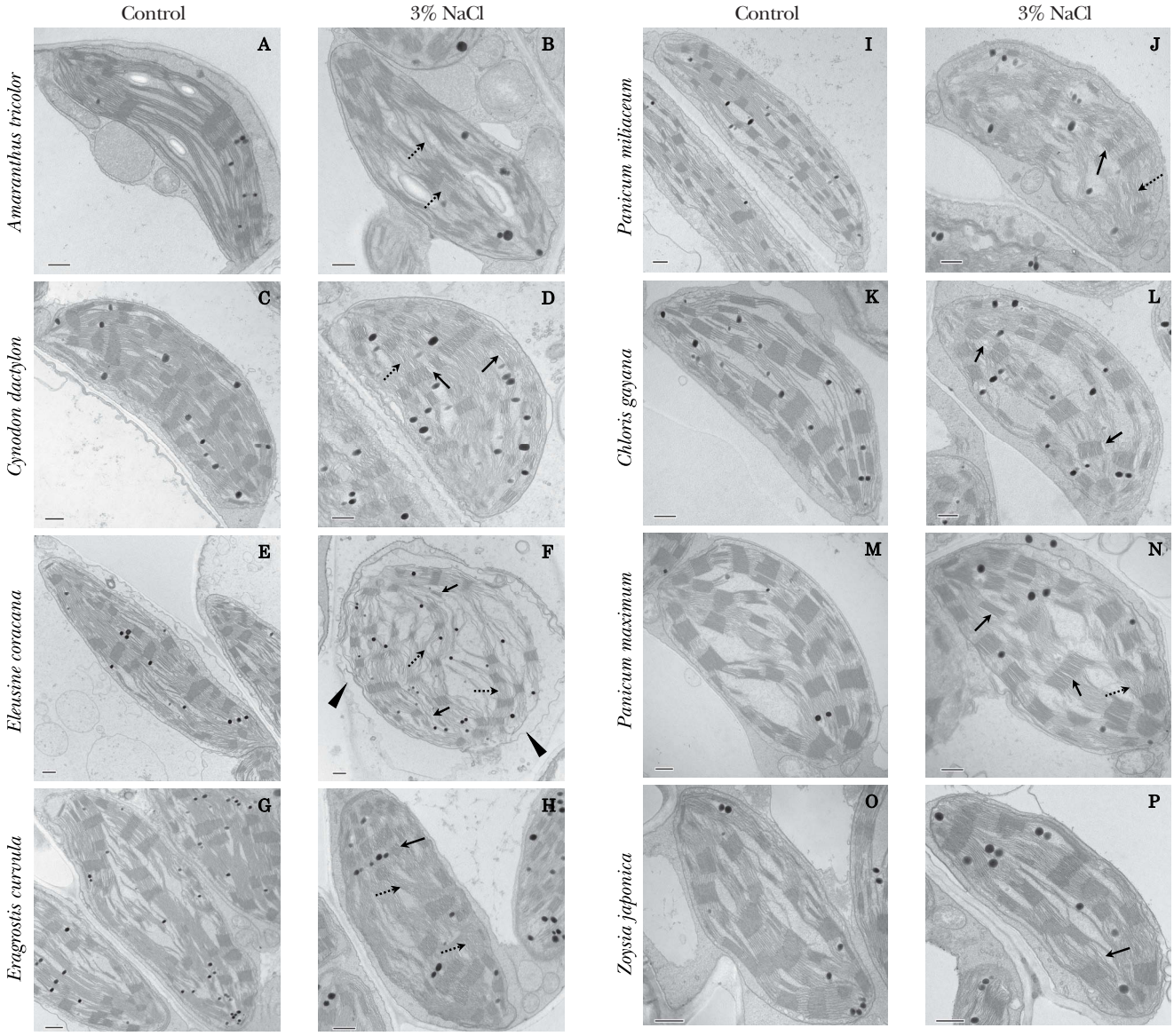

Fig. 3. Ultrastructure of MC chloroplasts in the middle part of the leaves. MC chloroplasts of control and 3% NaCl-treated plants in *Amaranthus tricolor* (A and B), *Cynodon dactylon* (C and D), *Eleusine coracana* (E and F), *Eragrostis curvula* (G and H), *Panicum miliaceum* (I and J), *Chloris gayana* (K and L), *Panicum maximum* (M and N) and *Zoysia japonica* (O and P) are shown. Solid, dashed and triangular arrows show swelling of thylakoids, undulation of thylakoid membranes and disruption of envelopes, respectively. Bars = 0.5 μm.

observed under a TEM (Hitachi H-7500) at 100 kV.

4. Quantitative parameters of chloroplast properties

The length of appressed and non-appressed thylakoids, the number of thylakoids per granum, and stromal and starch areas were estimated with the ImageJ program, a free, Java-based image-processing package (Rasband, 1997–2007). Granal index, stromal area and appressed thylakoid density were calculated as described previously (Omoto et al., 2009). In each species, at least 6 chloroplasts from three different plants were investigated.

5. Statistic analysis

Data obtained from the experiments were statistically analyzed by ANOVA and Scheffe's test. All values were expressed as mean ± SE.

Results

1. Plant growth

Salt treatment for 5 d suppressed the plant growth and significantly reduced the shoot biomass (Fig. 1). In both subtypes, the dry weights of salt-treated plants were decreased by about 40–70% except for *Z. japonica* whose dry weight was decreased by only 23%. In *C. gayana*, salt secretion was observed onto the surface of the leaf blades during salt treatment (data not shown).

2. Chlorophyll content

As shown in Fig. 2, salinity stress caused a significant decline in leaf chlorophyll content. The most severely salt-affected species was *P. maximum*, whereas the most unaffected species was *Z. japonica*. Their chlorophyll contents were decreased by 49 and 13%, respectively. In the other species, the chlorophyll content of salt-treated plants decreased by 20–30% in comparison with the control.

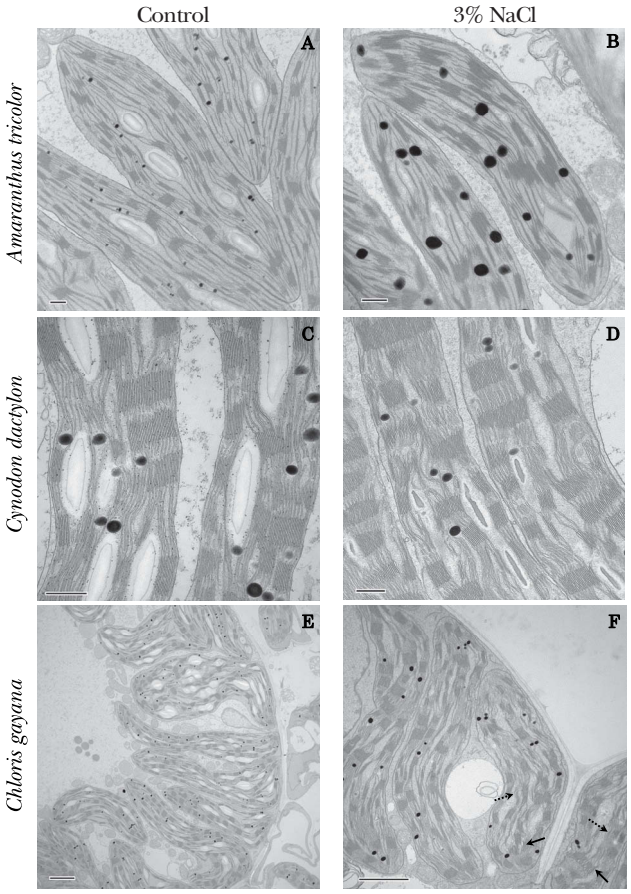

Fig. 4. Ultrastructure of BSC chloroplasts in the middle part of the leaves. BSC chloroplasts of control and 3% NaCl-treated plants in *Amaranthus tricolor* (A and B), *Cynodon dactylon* (C and D) and *Chloris gayana* (E and F) are shown. Solid and dashed arrows show swelling of thylakoids and undulation of thylakoid membranes, respectively. Bars (A, B, C and D) = 0.5 μm. Bars (E and F) = 2 μm.

3. Chloroplast ultrastructure

Mesophyll cell chloroplasts of control plants exhibited lens-shaped structure, with a typical arrangement of grana and stroma thylakoids (Fig. 3A, C, E, G, I, K, M, O). However, MC chloroplasts of salt-treated plants showed structural alterations (Fig. 3B, D, F, H, J, L, N, P); swelling of thylakoids, undulation of thylakoid membranes and disruption of envelopes. The most severely-damaged species was *E. coracana*; the shape of its MC chloroplasts was irregular in comparison with that of control (Fig. 3E, F). In contrast, the most unaffected species was *Z. japonica*, whose MC chloroplasts were structurally not different from those of control (Fig. 3O, P).

Figure 4 shows BSC chloroplasts of *A. tricolor*, *C. dactylon* and *C. gayana*. BSC chloroplasts of control plants had well-developed grana as in MC chloroplasts and did not show any abnormalities in their ultrastructures (Fig. 4A, C, E). Unlike MC chloroplasts, BSC chloroplasts were hardly damaged by salinity stress (Fig. 4B, D, F). The structure was almost similar to that in control; the grana thylakoids were packed closely, and no disruption of envelope was noticed (Fig. 4B, D, F) although swelling of thylakoids and undulation of thylakoid membranes (Fig. 4F) were observed on rare occasions. We also examined BSC chloroplasts of salt-treated *E. coracana*, *E. curvula*, *P. miliaceum*, *P. maximum* and *Z. japonica*, and obtained comparable results as shown in Fig. 4B, D, F (data not shown). The size of plastoglobuli was larger in salt-treated *A. tricolor* than in control (Fig. 4B).

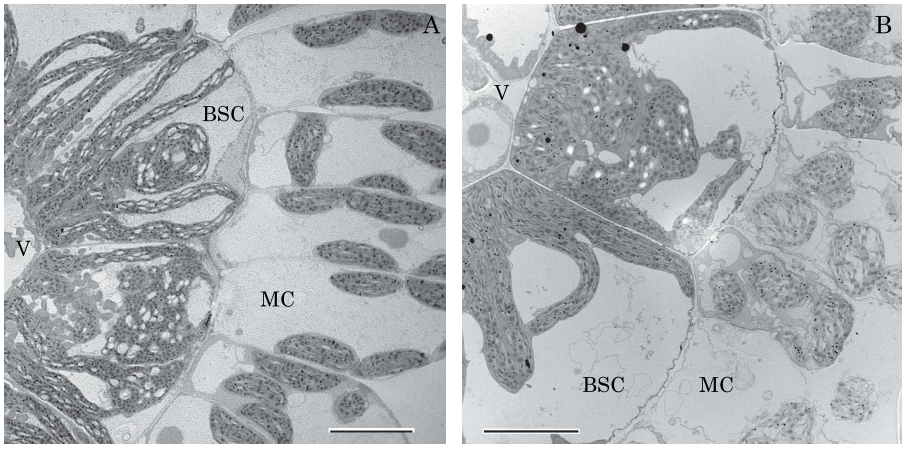

Fig. 5. Low magnification view of the middle part of mature fourth leaf blades in *Eleusine coracana* with and without 3% NaCl treatment for 5 d. A, Control plant. B, 3% NaCl-treated plant. BSC, bundle sheath cell; MC, mesophyll cell; V, vascular bundle. Bars = 10 μm.

Table 1. Parameters of granal development in BSC chloroplasts of NAD-ME and PCK type C_4 plants.

Species	No. of thylakoids per granum	Granal index (%)	Appressed thylakoid density ($\mu m\ \mu m^{-2}$)	Stromal area (μm^2)	Starch area (μm^2)
			Control		
NAD-ME type					
Amaranthus tricolor	5.3±0.2	71.2±1.8	7.6±0.6	23.3±1.5	1.9±0.2
Cynodon dactylon	6.4±0.1	81.7±0.6	6.8±0.6	60.8±4.6	14.6±1.0
Eleusine coracana	6.9±0.2	59.8±0.9	5.3±0.6	56.9±2.9	3.9±0.4
Eragrostis curvula	6.7±0.1	64.6±1.8	9.1±0.6	37.4±2.0	4.0±0.5
Panicum miliaceum	8.0±0.1	54.9±0.2	6.5±0.2	54.6±3.5	9.1±1.2
PCK type					
Chloris gayana	6.0±0.2	66.1±1.2	7.2±0.7	34.6±2.1	2.5±0.3
Panicum maximum	7.0±0.2	69.4±0.2	8.7±0.4	62.1±2.3	6.7±1.4
Zoysia japonica	9.1±0.3	65.6±0.9	13.3±0.2	19.8±1.0	2.6±0.4
			3% NaCl		
NAD-ME type					
Amaranthus tricolor	7.3±0.2**	75.8±0.5	9.8±0.2*	18.3±0.8**	1.1±0.3
Cynodon dactylon	8.1±0.2**	85.3±0.2	9.5±1.2	34.6±3.4**	0.9±0.2**
Eleusine coracana	7.4±0.2	67.1±0.2*	7.2±1.3	44.3±3.1**	0.7±0.2**
Eragrostis curvula	7.4±0.2*	74.3±0.7*	12.6±1.1*	27.8±1.7**	1.0±0.3**
Panicum miliaceum	9.4±0.2**	78.0±0.9**	12.0±0.6**	42.9±2.6*	2.2±0.3**
PCK type					
Chloris gayana	6.7±0.1**	70.5±0.7	8.2±0.6	26.0±1.2**	0.5±0.1**
Panicum maximum	7.9±0.2*	70.1±0.3	8.5±0.4	32.4±1.0**	1.5±0.2**
Zoysia japonica	8.7±0.3	69.7±0.8	16.0±0.9	15.2±0.6**	0.5±0.2**

Granal index, the percentage of the length of all appressed thylakoid membranes to the total length of all thylakoid membranes. Appressed thylakoid density, the length of appressed thylakoid membranes per stromal area. Stromal area, the total area of the chloroplast minus the area of starch granules. Stromal and starch areas represent mean area per chloroplast. Values are means±SE from at least 6 BSC chloroplasts from 3 different plants. * and ** indicate significant differences from control at $P<0.05$ and $P<0.01$, respectively.

Figure 5 shows low magnification views of *E. coracana*. Swelling of chloroplasts and disruption of envelopes and thylakoids were observed only in MC (Fig. 5B). The striking difference in chloroplast structure in BSC was not observed between control and salt-treated plant (Fig. 5A, B). Less starch was accumulated in BSC chloroplasts of salt-treated plant than in those of the control; however, the accumulation varied among chloroplasts for reasons not clearly understood. These figures clearly suggest that chloroplasts of MC are more sensitive to salinity stress than those of BSC.

4. Quantitative properties of chloroplasts

Because we were not able to decide whether salinity induced granal development by TEM observation, we examined the change in granal quantity in BSC chloroplasts. In all species except *Z. japonica*, the number of thylakoids per granum in salt-treated plants was higher than that in control (Table 1). The value in *Z. japonica* was slightly decreased by salt treatment although the decrease was not significant (Table 1). Granal indices and appressed thylakoid density were generally higher in salt-treated plants than in the control. Especially *P. miliaceum* showed highly significant increase ($P<0.01$). *C. dactylon* and the three species belonging to PCK subtype showed no significant differences in either parameter (Table 1). In all the species, stromal and starch areas were considerably decreased by salinity stress (Table 1).

The number of thylakoids per granum in MC chloroplasts was investigated. The values in *A. tricolor*, *E. curvula*, *C. gayana*, *P. maximum* and *Z. japonica* were not different between control and salt-treated plants (Table 2). The values in *C. dactylon*, *E. coracana* and *P. miliaceum* were not estimated in salt-treated plants due to severe damage on grana thylakoids, which was more prominent in the grana with fewer stackings (Table 2).

Discussion

Salinity-induced granal development in BSC chloroplasts of NADP-ME type C_4 species was previously demonstrated by Hasan et al. (2005, 2006) and Omoto et al. (2009). However, it remains to be elucidated whether this

Table 2. Number of thylakoids per granum in MC chloroplasts of NAD-ME and PCK type C_4 plants.

Species	No. of thylakoids per granum	
	Control	3% NaCl
NAD-ME type		
Amaranthus tricolor	4.3±0.3	4.4±0.2
Cynodon dactylon	7.2±0.4	ND
Eleusine coracana	6.8±0.2	ND
Eragrostis curvula	7.3±0.3	7.4±0.3
Panicum miliaceum	7.3±0.3	ND
PCK type		
Chloris gayana	8.7±0.3	8.7±0.3
Panicum maximum	9.0±0.2	8.7±0.4
Zoysia japonica	7.8±0.3	8.0±0.6

Values are means±SE from ten MC chloroplasts. ND, not determined due to severe damage.

phenomenon is general in C_4 plants, including NAD-ME and PCK type species. In this work, we investigated several species which belong to NAD-ME and PCK subtypes. We initially examined the shoot dry weight and chlorophyll content to know how salinity stress affected the plant growth. In all species, both shoot dry weight (Fig. 1) and chlorophyll content (Fig. 2) decreased in salt-treated plants. Thus, the salinity treatment exerted negative effects on the growth in all species. However, the decrease of both values in *Z. japonica* was less than that in other species (Figs. 1, 2); hence, it is considered that *Z. japonica* has a higher salinity resistance.

Salinity caused damages on the structure of chloroplasts, but the degree was more marked in MC than in BSC. The structural changes were particularly prominent in thylakoids. In all species, most MC chloroplasts in salt-treated plants exhibited thylakoid swelling and undulation of thylakoid membranes (Fig. 3B, D, F, H, J, L, N, P). In addition, MC chloroplasts of salt-treated *E. coracana* exhibited disruption of chloroplast-envelope (Fig. 3F). MC chloroplasts of *Z. japonica* were well preserved under salinity condition compared to those of other species (Fig. 3P). As described above, the shoot dry weight and chlorophyll content of *Z. japonica* were hardly affected by salinity stress; hence their chloroplasts were hardly damaged.

In all species, almost no marked damages were observed in BSC chloroplasts after salt treatment (Fig. 4). The undulation of thylakoid membranes and the swelling of thylakoids were observed on rare occasions (Fig. 4F), but the basic structural integrity was preserved. Since the low sensitivity of BSC chloroplasts and the high sensitivity of MC chloroplasts to salinity stress were previously reported in maize and several other species belonging to NADP-ME type (Hasan et al., 2005, 2006; Omoto et al., 2009), this may be a common feature in all C_4 plants regardless of subtype.

Hasan et al. (2005) suggested that the degradation of MC chloroplasts by salinity stress in NADP-ME type species was light-dependent and associated with the generation of reactive oxygen species (ROS). They also suggested that the difference in granal development between BSC and MC chloroplasts are related to the differential sensitivity of these chloroplasts to salinity stress. BSC chloroplasts of NADP-ME type species generally lack grana (Ghirardi and Melis, 1983) and are limited in photosystem Ⅱ(PSⅡ) activity (Meyerhoff and Westhoff, 1993; Romanowska et al., 2006). Therefore, Hasan et al. (2005) considered that the production of ROS is low in BSC chloroplasts and accordingly the BSC chloroplasts are less damaged by salinity. Since BSC chloroplasts of NAD-ME and PCK type species contain well-developed grana as MC chloroplasts, we had supposed that both chloroplasts could generate ROS and hence be damaged at the same level. However, the results were different from our presumption. The reason for the difference in salt sensitivity between BSC and MC chloroplasts remains unknown, but possible interpretations have been suggested. Furumoto et al. (2000) found that some stress-inducible genes, such as a salt-inducible gene encoding glycine-rich RNA-binding protein (GR-RBP) were expressed specifically in BSC in maize. It has been considered that GR-RBPs act for stabilization of mRNA, and would lead to the accumulation of stress-related proteins and the secondary metabolites having protective functions (Lorković, 2009). Kim et al. (2007) showed that catalase and peroxidase activities were increased by expression of GR-RBP in *Arabidopsis* under cold stress. In NAD-ME and PCK type species, whether the GR-RBP is expressed by salinity and whether it is localized specifically in BSC remain unknown. The investigation on the expression pattern of GR-RBP in NAD-ME and PCK type species would present a clue to clarify the differential sensitivity between MC and BSC chloroplasts in C_4 plants.

A visible change in grana structure of BSC chloroplasts by salinity was not easily detected by TEM observation, but the image analysis revealed a quantitative increase of grana (Table 1). Three parameters, number of thylakoids per granum, granal index and appressed thylakoid density, suggested an induction of granal development in BSC chloroplasts by salinity. However, *Z. japonica* showed no significant difference in any parameters between control and salt-treated plants (Table 1). In our previous study, almost all species belonging to NADP-ME type showed significant increase in these parameters under salinity (Omoto et al., 2009); however, the granal development in NAD-ME and PCK type species was not so pronounced as that in NADP-ME type species. Hasan et al. (2006) suggested that the granal development in BSC chloroplasts of maize might contribute to the increase in PS Ⅱ activity

in BSC of salt-treated plants because the photosynthetic activity in MC may be diminished due to the structural damages. Unlike NADP-ME type species, BSC chloroplasts of NAD-ME and PCK type species have inherently well-developed grana (Hattersley and Watson, 1992) and show PSII activity as in MC chloroplasts (Romanowska and Drożak, 2006). We therefore suggest that in these subtypes the grana of BSC chloroplasts are also increased according to the damage of MC chloroplasts, but there may not be much necessity to promote further granal development as in NADP-ME type species.

The number of thylakoids per granum in MC chloroplasts was investigated to find an association with granal development in BSC chloroplasts. The values in *A. tricolor*, *E. curvula*, *C. gayana*, *P. maximum* and *Z. japonica* showed no significant difference between control and salt-treated plants, whereas those in *C. dactylon*, *E. coracana* and *P. miliaceum* were not estimated in treated plants due to severe damage of garanal thylakoids (Table 2). These results suggested that grana stacking in MC chloroplasts was not changed by salinity, and was disrupted when the stress exceeded the threshold level. The granal development in BSC chloroplasts of salt-treated plants, therefore, may be a reaction to make up for the loss of PSII activity in MC chloroplasts.

Several studies have reported the changes of granal stacking by the alternation of nutrient concentrations. Barber (1982) and Bernal et al. (2006) showed that excess Na and Cu induced granal development. Lidon and Teixeira (2000) and Molas (2002) showed that excess Mn and Ni decreased the growth of grana. The mechanisms of grana formation have not yet been fully understood. Barber (1986) suggested that surface electrical changes control the grana formation. Navari-Izzo et al. (2000) suggested that the rise in the protein-to-lipid ratio may play a role in the increase in granal stacking. However, we can not explain the reason why the grana of BSC chloroplasts were developed by salinity but those of MC chloroplasts were not. Further studies are necessary to clarify the mechanism.

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