Salinity Stress Induces Granal Development in Bundle Sheath Chloroplasts of Maize, an NADP-Malic Enzyme-Type C₄ Plant

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Abstract: Zea mays is an NADP-malic enzyme (ME)-type C₄ plant. The C₄ plants of this type are attractive species for ultrastructural and physiological studies because they possess reduced grana in bundle sheath cell (BSC) chloroplasts. The present study evaluated the effect of salinity on granal development in BSC chloroplasts of maize. The plants were grown in soil media and after the second leaf was fully developed they were irrigated with four different concentrations (0, 1, 2 and 3%) of NaCl for 5 d. Ultrastructure, quantitative properties of chloroplasts and chlorophyll fluorescence parameters were evaluated. Granal stacking in BSC chloroplasts was induced by treatment with 2 or 3% NaCl. In contrast, granal stacking in mesophyll cell (MC) chloroplasts was reduced and disorganized by the NaCl treatment due to swelling of thylakoid. In control plants, only 2% of grana in BSC chloroplasts contained more than three thylakoids. In the plants treated with 3% NaCl, however, 66% of grana contained more than three thylakoids in BSC chloroplasts. The maximum number of thylakoids in grana of BSC chloroplasts in the control and 3% NaCl-treated plants, was 4 and 16 respectively. The granal index in BSC chloroplasts of 3% NaCl-treated plants was more than three times higher than that in the control plants. Chlorophyll fluorescence parameter analysis showed that the maximal quantum yield (Fv/Fm), the effective quantum yield of PSII (ΦPSII) and PSII-driven electron transport rate (ETR) decreased with the increase of salinity stress. These results suggest that the suppression mechanism of granal development in BSC chloroplasts of maize is influenced by salinity.

Key words: Bundle sheath, Chlorophyll fluorescence, Chloroplasts, C₄ plant, NADP-malic enzyme, Salinity, Zea mays L.

C₄ plants have higher photosynthetic efficiency and lower photorespiration rate than C₃ plants. C₄ plants can utilize a low concentration of CO₂ and have a permeability barrier to diffusion of CO₂ out of bundle sheath cell (BSC), where Rubisco is located exclusively (Hatch, 1987; Jenkins et al., 1989; Henderson et al., 1992). In these plants, CO₂ is initially fixed into C₄ acids in the mesophyll cell (MC), which subsequently diffuse to the BSC and undergo decarboxylation to release CO₂ which enters the C₃ pathway (Hatch, 1987). Based on the enzyme that catalyzes the respective decarboxylation of C₄ acids in BSC, C₄ plants are separated into three subgroups: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PEP-CK) types. Each type of C₄ plant shows differences not only in the structure and arrangement of BSC, but also in biochemical properties and in the intercellular transport of metabolites between MC and BSC (Gutierrez et al., 1974; Hatch, 1987; Leegood, 2002).

Maize, one of the most important crops in the human diet and agro-industrial purposes worldwide, is a typical C₄ plant belonging to the NADP-ME type. It exhibits a Kranz-type anatomy of leaves and possesses dimorphic chloroplasts differing in structure and function (Malkin and Niogy, 2000). Chloroplasts in MC possess well-developed grana and both PSI and PSII. In contrast, chloroplasts in BSC are characterized by highly reduced grana and deficiency in PSII activity (Meierhoff and Westhoff, 1993; Malkin and Niogy, 2000). Among some species of NADP-ME type of C₄ plants such as Japanese-millet (Echinochloa utilis),
Eriachne aristidea, Salsola richteri and Haloxylon persicum, maize has the most reduced granal stacking in BSC chloroplasts (Voznesenskaya et al., 1999; Taniguchi et al., 2003).

Salinity stress is known to be mediated by an enhanced generation of reactive oxygen species (ROS) (Greenway and Munn, 1980; Hernandez et al., 2001; Alscher et al., 2002; Rios-Gonzales et al., 2002; Yamane et al., 2003). Chloroplasts are one of the most sensitive systems to various stress factors due to the fact that chloroplasts are recognized as one of the major sources of ROS production in the plant cell (Dat et al., 2000; La Rocca et al., 2001; Pechova et al., 2003). Due to deficiency in PSII activity, BSC chloroplasts in maize may have a lower capacity than MC chloroplasts in producing ROS. Doulis et al. (1997) reported that maize leaves have differential localization of antioxidant enzymes in MC and BSC. This fact might be related with higher sensitivity of chloroplasts in MC to oxidative stresses induced by herbicide (Pechova et al., 2003), ABA (Hu et al., 2005) and salinity (Hasan et al., 2005). In addition, our previous observation suggested that the thickness of grana is increased by salinity although detailed analysis was not conducted (Hasan et al., 2005). The changes in structure and photosynthetic characteristics of chloroplasts in MC of maize during leaf development have been well documented (Wang and Hu, 1988; Kutik et al., 1999; Kutik et al., 2001; Kołodziejek et al., 2003). The decrease in granal development in MC chloroplasts of C, plant under Na-deficient condition has also been reported (Grof et al., 1989). However, the effects of salinity on the granal structure of BSC chloroplasts have not been examined in detail.

Ultrastructural studies on the leaves of the plants exposed to salinity stress revealed that the salinity-induced damages in chloroplasts are dependent on light (Mitsuya et al., 2003; Hasan et al., 2005) and are mediated by ROS (Yamane et al., 2004). The damages to MC and BSC chloroplasts in maize have been reported previously (Hasan et al., 2005). In the present study, we examined in detail the development of granal stacking in BSC chloroplasts of maize exposed to salinity stress. We also analyzed the parameters of chlorophyll fluorescence to monitor the physiological response of photosynthetic apparatus to salinity stress.

Materials and Methods

1. Plant material

Maize (Zea mays L. ’Golden Bantam’) plants were grown as described previously (Hasan et al., 2005). We started the salt treatment when the second leaf blades (coleoptile was numbered as leaf zero) of the plants were fully developed. The plants were irrigated with 50 ml of 0 (control), 1, 2 and 3% NaCl solutions daily. Five days after the start of NaCl treatment at which the visible symptoms were observed in the 2 and 3% NaCl, the samples were collected to analyze chloroplast ultrastructure and chlorophyll fluorescence parameters.

2. Transmission electron microscopy

Small portions (1×2 mm) of the middle part of the second leaf were prepared for ultrastructure study as described previously (Hasan et al., 2005). Samples were examined with a transmission electron microscope (Hitachi H-7500, Japan) at 100 kV. Photographs of MC and BSC chloroplasts from each sample were taken for quantitative analysis.

3. Quantitative parameters of chloroplast properties

The chloroplast area, starch area, the length of thylakoids and the number of thylakoids per granum were estimated with an Image Analysis program (Image Tool for Windows Version 3.00, UTHSCSA, USA) in at least ten chloroplasts from three different plants. Granal index and thylakoid density were estimated according to Voznesenskaya et al. (1999). Relative distribution of thylakoid numbers per granum was calculated as follows:

\[
\text{number of grana with respective number of thylakoids in a chloroplast} \times \frac{\text{total number of grana in a chloroplast}}{100} \%
\]

Granal index, the density of appressed thylakoids and non-appressed thylakoids of MC chloroplasts in the 2 and 3% NaCl were not examined because the thylakoid membrane was severely distorted.

4. Chlorophyll fluorescence

Chlorophyll fluorescence was measured with a portable chlorophyll fluorometer (PAM-2100, Waltz, Effeltrich, Germany) in the same area of the second leaf blades as used for transmission electron microscopy. After dark adaptation of plants for 1 h, the minimal fluorescence (Fo) was measured with weak modulated irradiation. Thereafter, 400-ms saturating flash was applied to obtain the maximum fluorescence yield (Fm). Thus, the maximal quantum yield of dark adapted leaf (Fv/Fm = (Fm−Fo)/Fm) was obtained (Schreiber et al., 1986). Immediately after the leaf was irradiated with actinic beams (665 nm) and at 20 s intervals, saturated flash was applied to record Fm’. After chlorophyll fluorescence steady state was reached and Fo was recorded, actinic irradiation was removed and far-red irradiation (730 nm) was given to record Fo’. The effective quantum yield of PSII, \( \Phi_{\text{PSII}} = (Fm'−Fo')/Fm' \); coefficient of photochemical fluorescence quenching, \( qP = (Fm'−Ft)/(Fm'−Fo') \); coefficient of non-photochemical fluorescence quenching, \( qN = (Fm−Fm')/(Fm−Fo') \); efficiency of excitation energy capture by open PSI reaction centre, \( Fv'/Fm' = (Fm'−Fo')/Fm' \); and PSII-driven electron transport rate,
Fig. 1. Ultrastructure of chloroplasts in mesophyll cell in control and NaCl-treated plants. A. Chloroplast in mesophyll cell from control plant showing well organized grana. B. Chloroplast in mesophyll cell from 1% NaCl-treated plant. The structure is almost similar to control. C. Chloroplast in mesophyll cell from 2% NaCl-treated plant shows swollen thylakoid. D. Chloroplast in mesophyll cell from 3% NaCl-treated plants. The damage is severer than that in 2% NaCl-treated plants. Arrows show granal stacking. p = plastoglobuli, s = starch granules. Bar = 1 µm.
Fig. 2. Ultrastructure of chloroplasts in bundle sheath cell of control and NaCl-treated plants. A. Chloroplast in bundle sheath cell from control plant showing abundant starch and rudimentary grana. B. Chloroplast in bundle sheath cell from 1% NaCl-treated plant. C and D. Chloroplasts in bundle sheath cell from 2 and 3% NaCl-treated plants, respectively, with developed granal stacking. Arrows show granal stacking in bundle sheath chloroplasts. p = plastoglobuli, s = starch granules. Bar = 1 µm.
ETR = \frac{(Fm'−Ft)}{Fm'} \cdot I \cdot f \cdot \alpha

where $I$ is photosynthetic photon flux density (PPFD), $f$ is the fraction of absorbed quanta used by PSII and $\alpha$ is the leaf absorbance. The fraction of absorbed quanta used by PSII for C$_4$ plants was assumed to be 0.4 (von Caemmerer, 2000) and the leaf absorbance for maize was assumed to be 0.87 (Naidu and Long, 2004).

In addition, three derived chlorophyll fluorescence parameters were estimated to analyze the allocation of fraction of excitation energy. They were the fraction of photon energy absorbed by PSI antennae and dissipated via thermal energy in the antenna, $D = 1 - \frac{Fv'}{Fm'}$; the fraction of photon energy absorbed by PSI antennae utilized for photosynthetic electron transport, $P = qP \times \frac{Fv'}{Fm'}$; and the fraction of excess excitation energy neither dissipated in the PSI antennae nor utilized for photochemistry, $Ex = Fv' / Fm' \times (1 - qP)$ (Demmig-Adams et al., 1996). For each treatment, measurements were made on five different plants.

5. **Statistical analysis**

Data were statistically analyzed using ANOVA followed by Dunnett’s Multiple Comparison Test. Significant difference was analyzed based on P-value < 0.05.

**Results**

1. **Chloroplast ultrastructure**

We examined the structure of chloroplasts of maize plants irrigated with 0, 1, 2 or 3% NaCl. Fig. 1A-D show the chloroplast ultrastructure of MC from the second leaf blades of control plants and the NaCl-treated plants. The chloroplasts in MC of the control plants (Fig. 1A) were characterized by well-organized thylakoid membranes and possessed well-developed grana and some starch granules. The structure of the chloroplasts in MC was damaged by NaCl showing a gradual increase in the structural damage with increasing NaCl concentration as indicated by swelling and disorganizing of thylakoid membrane (Fig. 1B-D).

Fig. 2A-D show BSC chloroplasts of the 0 (control), 1, 2 and 3% NaCl-treated plants. Chloroplasts in BSC of control plants show a typical structure of NADP-ME type of C$_4$ plant, in which thylakoids were not appressed so much, grana were rudimentary and the starch granules were abundant. In the NaCl-treated plants, the chloroplasts in BSC were structurally preserved (Fig. 2B-D). The size of chloroplasts and the number and size of starch granules were decreased (Table 1), but there was no structural distortion in the thylakoid and the envelope. Thus, the structure of BSC chloroplasts were less affected by salt stress. However, the development of granal stacking in BSC chloroplasts was induced by salinity (Fig. 2C,D). In the 2 and 3% NaCl-treated plants, the granal stacking in BSC chloroplasts was promoted, while the thylakoids in MC chloroplasts were severely damaged (Fig. 1C,D and 2C,D).

Fig. 3 represents the distribution of thylakoid number per granum in MC and BSC chloroplasts of control and NaCl-treated plants. Number of thylakoids per granum in MC chloroplasts tended to decrease with the increase of NaCl concentration. Fractions of grana containing more than 16 thylakoids were

| Table 1. Parameters of granal development, chloroplast area and starch area in mesophyll and bundle sheath chloroplasts of the control and NaCl-treated plants. |
|---|
| **Treatment** | **Granal index** | **Appressed thylakoid density** | **Non-appressed thylakoid density** | **Thylakoid density** | **Chloroplast area** | **Starch area** |
| | (%) | (µm $\mu$m$^{-2}$) | (µm $\mu$m$^{-2}$) | (µm $\mu$m$^{-2}$) | (µm$^2$) | (µm$^2$) |
| **Mesophyll chloroplasts** | | | | | | |
| Control | 26.5 ± 4.2 | 16.0 ± 2.1 | 43.8 ± 5.2 | 59.8 ± 6.4 | 19.1 ± 1.1 | 0.1 ± 0.0 |
| 1% NaCl | 24.4 ± 3.1* | 12.4 ± 1.6 | 39.1 ± 4.7 | 51.5 ± 6.6 | 19.4 ± 2.4 | 0.1 ± 0.0 |
| 2% NaCl | nd | nd | nd | nd | 18.6 ± 1.8 | 0.0 ± 0.0* |
| 3% NaCl | nd | nd | nd | nd | 18.5 ± 1.6† | 0.0 ± 0.0*† |
| **Bundle sheath chloroplasts** | | | | | | |
| Control | 6.8 ± 1.2 | 1.2 ± 0.2 | 16.9 ± 2.2 | 18.1 ± 2.3 | 19.7 ± 2.6† | 10.2 ± 1.8† |
| 1% NaCl | 9.4 ± 1.0 | 1.8 ± 2.5 | 16.1 ± 2.4 | 18.8 ± 3.4 | 18.9 ± 2.4 | 5.2 ± 0.7* |
| 2% NaCl | 18.8 ± 2.6* | 3.2 ± 0.5* | 13.9 ± 2.0 | 17.1 ± 3.7 | 10.0 ± 1.8* | 2.8 ± 0.4** |
| 3% NaCl | 31.2 ± 5.5** | 5.6 ± 1.8** | 12.0 ± 0.8 | 17.6 ± 0.9 | 8.7 ± 2.0*D | 2.8 ± 0.7**D |

Values are means ± s.e. of ten or more chloroplasts. * = significantly different from control (p<0.05), ** = significantly different from control (p<0.01), †, ‡, ‡‡, †, ‡, ‡‡, ††, ‡‡‡, ††† adopted from Hasan et al. (2005).
significantly reduced by the treatment with 2 or 3% NaCl in MC chloroplasts, but the fractions of grana containing less than 9 thylakoids were increased (Fig. 3A). In contrast, the number of thylakoids per granum in BSC chloroplasts increased with the increase of NaCl concentration (Fig. 3B). More than 75% of grana in BSC chloroplasts in the control and 1% NaCl-treated plants contained only 2 thylakoids, but 64 and 80% of grana in BSC chloroplasts in the 2 and 3% NaCl-treated plants, respectively, contained more than 3 thylakoids. In BSC chloroplasts in the 3% NaCl-treated plants, only 20% of grana contained 2 thylakoids and more than 43% of grana contained more than 5 thylakoids. The maximum number of thylakoids in grana was 4, 6, 16 and 16 in BSC chloroplasts in the control, 1, 2 and 3% NaCl-treated plants, respectively.
Likewise, granal index and other parameters of granal development in BSC chloroplasts in the 3% NaCl-treated plants were higher than those of control (Table 1). Granal index of BSC chloroplasts of the control plants was 6.8. The granal index in the 2 and 3% NaCl-treated plants was 18.8 and 31.2, respectively, i.e., more than two and four times, respectively, that of the control. Appressed thylakoid density increased and non-appressed thylakoid density decreased with the increase of NaCl concentration. The thylakoid density was not affected by the NaCl treatment. The density of appressed and non-appressed thylakoids and the granal index of MC chloroplasts were not estimated due to severe damage of thylakoids in the 2 and 3% NaCl-treated plants (Table 1).

Fig. 4 The parameters of chlorophyll fluorescence in the second leaf blades of maize plant exposed to salinity. A. Fv/Fm, maximal efficiency of PSII photochemistry in the dark-adapted state. B. qP, coefficient of photochemical quenching. C. ΦPSII, effective quantum yield of PSII. D. qN, coefficient of non-photochemical fluorescence quenching. E. ETR, PSII-driven electron transport rate. F. P, fraction of photon energy absorbed in PSII antennae utilized for photosynthetic electron transport. G. D, fraction of photon energy absorbed in PSII antennae and dissipated via thermal energy in the antenna. H. Ex, fraction of excess excitation energy neither dissipated in the PSII antennae nor utilized for photochemistry. Values are means ± s.e. of 5 samples. ** = significantly different from control (p<0.01).
2. Effect of salinity on chlorophyll fluorescence parameters

Fig. 4A,C and E show that Fv/Fm, $\Phi_{\text{PSII}}$ and ETR decreased with the increase of NaCl concentration. Fv/Fm was reduced by 32 and 54% (significant at p<0.01) in the 2 and 3% NaCl-treated plants, respectively, as compared to that of control. $\Phi_{\text{PSII}}$ and ETR decreased by 54 and 67% by the treatment with 2 and 3% NaCl, respectively, significantly lower than those of control. Salinity tended to increase qN and decrease qP though not significantly (Fig. 4B,D). Meanwhile, P and Ex were significantly decreased (p<0.01) by salinity stress (Fig. 4F,H). As the consequence, D increased with the increase of salinity stress (Fig 4G).

Discussion

Salinity-induced structural changes of chloroplasts under light condition has been reported previously in C3 plants (Mitsuya et al., 2000, 2003; Rahman et al., 2000, under light condition has been reported previously in C3 plants (Mitsuya et al., 2000, 2003; Rahman et al., 2000). Reactive oxygen species (ROS) have been recognized as toxic compounds which mediate the damage of chloroplast possessing PSI and PSII activities and the non-cyclic electron flow. Hernandez et al. (1995) showed that the damage of chloroplasts isolated from pea leaves and exposed to NaCl was resulted from oxidative stress caused by ROS. However, in the present study, the structure of BSC chloroplasts in maize was relatively resistant to salinity stress. Even though the volume and the number of starch granules were decreased, the thylakoids were preserved under salinity stress. The weakness of the effect of salt stress on BSC chloroplasts may be related with a low productivity of ROS. Greatly reduced grana (Taniuchi et al., 2003) which correlated with deficiency in PSII activity (Meierhoff and Westhoff, 1993; Malkin and Niyogy, 2000) make BSC chloroplasts of maize restricted in the activity of non-cyclic electron flow and the production of ROS (Doulis et al., 1997). In addition, a spatial isolation from oxygen evolved in MC chloroplasts and its derivative ROS may have contributions to the resistance of BSC chloroplasts to salinity stress.

Due to reduced grana in BSC chloroplasts, MC chloroplasts in maize are the primary site to conduct PSII activity. The damage of MC chloroplasts (Fig. 1C,D) caused by the treatment with 2 or 3% NaCl was accompanied by the decrease of Fv/Fm, $\Phi_{\text{PSII}}$ and ETR (Fig. 4A,C,E). Fv/Fm is a parameter of the maximum quantum efficiency of PSII, and a decrease in this parameter is an indication of photoinhibitory damage in PSII reaction center (Maxwell and Johnson, 2000; Huang et al., 2004). The reduction in $\Phi_{\text{PSII}}$ indicates a decrease in the photosynthetic energy conversion in PSII center. This condition causes an increase in the amount of the reduced form of QA and therefore, reduced electron transfer driven by PSII (Stepien and Klobus, 2005). The decrease of Fv/Fm, $\Phi_{\text{PSII}}$ and ETR was reported as being accompanied by an enhanced production of ROS (Jin and Tao 2000; Stepien and Klobus, 2005). Light energy absorbed by light-harvesting complexes associated with PSII is allocated to one of three possible fractions. First, P, the fraction of excitation energy utilized to drive photosynthesis (photochemical reaction). Second, D, the fraction of excitation energy dissipated as heat (non-photochemical reaction). Third, Ex, a small portion of re-emitted fluorescence, the fraction of excitation energy neither utilized for photochemical process nor dissipated as heat (Demmig-Adams et al., 1996; Maxwell and Johnson, 2000). P and Ex were decreased drastically by the treatment with 2 and 3% NaCl (Fig. 4F,H). Thus, the major proportion of light energy absorbed by chlorophyll was dissipated as heat in NaCl-treated maize (Fig. 4D).

Ultrastructural data showed that granal stacking in BSC chloroplasts was increased (Fig. 2C,D) while that in MC chloroplasts was severely damaged by NaCl treatment (Fig. 1C,D) in maize plants. The damage of MC chloroplasts is light-dependent (for detail see Hasan et al., 2005). The increase of granal stacking in BSC chloroplasts may contribute to increase PSII activity of BSC in NaCl-treated plants as compared with that in the control plants. Under normal condition, PSII activity in maize almost exclusively located in MC due to reduced grana in BSC chloroplasts. Therefore, the fluorescence data in the 2 and 3% NaCl-treated plants might be a reflection of the parameters of PSII in both MC and BSC.

Fig. 2C,D show the increase of granal stacking in BSC chloroplasts of maize caused by salinity stress. The number of thylakoids per granum and granal index of BSC chloroplasts in the 2 and 3% NaCl-treated plants were significantly higher than those in the control plants (Fig. 3B and Table 1). These phenomena were accompanied with the severe damage of chloroplasts in MC (Fig. 1C,D). The activity of PSI is considered to be associated with the thickness of granal stacking in chloroplasts (Woo et al., 1970; Miller and Nobel, 1972; Meierhoff and Westhoff, 1993; Maxwell et al., 1999). We therefore suggest that granal development in BSC chloroplasts may be an adaptation to salinity where photosynthetic activity in MC was distorted due to severe damages in MC chloroplasts. However, the present data on chlorophyll fluorescence suggest that the granal development in BSC chloroplasts cannot recover effectively the overall activity of PSI and the non-cyclic electron transport in maize leaves.

Another possible interpretation on the granal development of BSC chloroplasts under salinity stress is the removal of the suppression mechanism of granal development in BSC chloroplasts. The granal development in BSC chloroplasts of NADP-ME type C4 plants is suppressed in from the early developmental stage of leaves when the Kranz anatomy is not yet
apparent (Nishioka et al., 1993). Since NaCl treatment in the present study was started after the target leaves had fully developed, a typical NADP-ME type C₄ plant structure was already established in these leaves. Therefore, the suppression mechanism of granal development is considered to be damaged by salinity stress. Some investigators reported that chloroplasts have a plasticity regarding stacked and unstacked thylakoid formation as adaptation to environmental changes (Miller and Nobel, 1972; Grof et al., 1989; Mullineaux, 2005). Maxwell et al. (1999) reported that granal stacking was reduced in mature leaf as acclimation to high light intensity. Staehelin and Arntzen (1983) described the physical mechanism which controls granal stacking. The formation of stacked membrane regions is largely mediated by mobile LHClI (light-harvesting chlorophyll complex II) particles. The ability of LHClI to form stacked regions is reduced by incorporation of negatively charged phosphate groups into the LHClI particles through protein phosphorylation. Therefore, NaCl may influence the phosphorylation status of LHClI.

Our findings would be useful to further investigate the suppression mechanism of granal development in BSC chloroplasts in the NADP-ME-type C₄ plants.

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