Strictness of the Centrifugal Location of Bundle Sheath Chloroplasts in Different NADP–ME Type C₄ Grasses

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Abstract: C₄ plants have many attractive traits for crops, but their structural and functional relationships are complicated. C₄ plants are different in bundle sheath cell (BSC) chloroplast location (centrifugal or centripetal) among species. The effects of light intensity on the centrifugal location of BSC chloroplasts were investigated in four grass species of NADP malic enzyme (NADP–ME) type (Zea mays, Echinochloa utilis, Sorghum bicolor and Eriachne aristida) by light and electron microscopy. Furthermore, the degree of granal development was examined to investigate the relation between BSC chloroplast location and dependence of BSC chloroplasts on the reducing power of mesophyll cells. We investigated BSC chloroplast location grown under high intensity light (HL) (600 μmol m⁻² s⁻¹) and low intensity light (LL) (2.5 μmol m⁻² s⁻¹) and dark conditions and counted the number of granal thylakoids per granum. Although BSC chloroplasts of maize maintained the centrifugal position under all light conditions, the centrifugal location of other species was disturbed under LL and in the dark. Granal development in BSC chloroplasts in the plants grown under HL was suppressed, although the suppression in Z. mays and S. bicolor was more prominent than in other two species. These findings indicate that there is a difference in the strictness of centrifugal location of BSC chloroplasts among NADP–ME type C₄ grass species and the strictness had no relation to the degree of granal development in BSC chloroplasts.

Key words: Bundle sheath chloroplast, C₄ plant, Centrifugal location, Echinochloa utilis, Eriachne aristida, NADP–ME type, Sorghum bicolor, Zea mays.

C₄ plants have a higher photosynthetic performance and water–nitrogen-use efficiencies than C₃ plants (Ehleringer and Monson, 1993; Leegood, 1997). Moreover, it has been indicated that C₄ plants have high resistances to salinity (Osmond et al., 1982). Thus, C₄ plants have attractive traits for crops, and the transfer of C₄ traits to C₃ plants is one strategy being adopted for improving the photosynthetic performance of C₃ crop species. For this aim, there had been many trials to express C₄ enzymes in C₃ crops with recombinant DNA technology ( Matsauka et al., 2001). However, researchers did not reach the goal of installation of a complete C₄ pathway into C₃ plants, because structural and functional relationships in C₄ plants are very complicated.

C₄ plants are classified into three subgroups depending on the major decarboxylation enzyme: NADP–malic enzyme (NADP–ME), NAD–malic enzyme (NAD–ME), and phosphoenolpyruvate carboxykinase (PEP–CK) type species (Downton, 1970; Gutierrez et al., 1974; Hatch et al., 1975). Differences among the three subtypes are observed not only in C₄ photosynthetic pathways but also in intracellular structures. The bundle sheath cell (BSC) chloroplasts of the three C₄ subtypes differ in development of grana and intracellular positions. BSC chloroplasts of the NADP–ME type species have rudimentary grana and have the centrifugal arrangement in monocots or the centripetal position in dicots. BSC chloroplasts of the NAD–ME type are granal and are arranged centripetally or centrifugally in monocots or centripetally in dicots. BSC chloroplasts of the PEP–CK type have well–developed grana and are centrifugal in arrangement.

The significance of the specific location of BSC chloroplasts observed in C₄ plants has been thought to be involved in metabolite transport and leakage of CO₂. C₄ photosynthesis is carried out by the coordinated function of BSC and MC. The C₄ photosynthetic enzymes are located separately in these cells, and the rapid exchange of metabolites between them is necessary for operation of the C₄ cycle. Therefore, the centrifugal position of BSC chloroplasts appears to be favorable for the exchange of photosynthetic intermediates (Hatch and Slack, 1970). Although C₄ acids are decarboxylated in BSC chloroplasts, mitochondria or the cytoplasm, the place of decarboxylation close to MC is under risk of CO₂ leakage. Therefore, the C₄ plants which have a centrifugal position of chloroplasts are thought to develop suberized lamella between BSC and MC for preventing the leakage (Ohsugi et al., 1988). On the other hand, the C₄ plants without suberized lamella (all C₄ dicots and some NAD–ME type grasses) have centripetal arrangement of BSC chloroplasts.
chloroplasts and mitochondria to increase resistance to CO₂ leakage by increasing the path length for diffusion (Hattersley and Browning, 1981).

No work has focused on the effect of light on the species-specific location of BSC chloroplasts in C₄ plants. The centripetal disposition of BSC chloroplasts in finger millet, an NAD- ME type C₄ plant, is related to the stage of tissue development but not to the chloroplast development, because light is not required for this positioning (Miyake and Yamamoto, 1987). The centripetal disposition of BSC chloroplasts requires cessation of leaf elongation, cytoplasmic protein synthesis and the formation of microfilaments (Miyake and Nakamura, 1993). However, no detailed investigation was conducted on other C₄ species.

In the present study, we examined the effect of light intensity on the centrifugal location of BSC chloroplasts in different NADP-ME type grass species. Such information will contribute to the understanding of the functional significance of the specific location of BSC chloroplasts. Furthermore, we examined the degree of granal development to discuss whether granal development of BSC chloroplasts could influence specific location of BSC chloroplasts.

Materials and Methods

1. Plant materials

Maize (Zea mays L. cv. Honey Bantam), Japanese-millet (Echinochloa utilis Ohwi et Yabuno cv. King Millet), sorghum (Sorghum bicolor Moench), and Eriachne (Eriachne aristidea F. Muell) were used as plant materials. We used Eriachne because it has been reported to be an NADP- ME type C₄ plant with developed grana in BSC chloroplasts (Prendergast et al., 1987). Following germination, these plants were grown for 2 weeks in a growth chamber with 12 h of illumination (600 µmol m⁻² s⁻¹ (high intensity light condition: HL), 2.5 µmol m⁻² s⁻¹ (low intensity light condition: LL)) at 30°C and 12 h of darkness at 25°C, or under continuous darkness. Fluorescent lamps were used as light source and plants were covered with mesh seats to make different light conditions. Maize and Eriachne were randomly oriented in the cells under LL condition. Although maize maintained the centrifugal arrangement of BSC chloroplasts (Fig. 1B), the centrifugal positioning of BSC chloroplasts of Eriachne in the mature leaf was disturbed (Figs. 1E, H and K). BSC chloroplasts of Japanese-millet (Fig. 1E) and sorghum (Fig. 1H) distributed to the adaxial side facing the light source.

2. Light and electron microscopy

Leaf tissues were fixed in Karnovsky’s fixative solution (Karnovsky, 1965) containing 0.05 M phosphate buffer (pH 7.2) and successively fixed in 2% osmium tetroxide dissolved in the same buffer. Samples were dehydrated in a series of graded acetone and propylene oxide, and embedded in Spurr’s resin (Spurr, 1969). Semithin and ultrathin sections were cut with glass or diamond knives on Ultracut-N microtome (Reichert Nissel). The semithin sections were stained with toluidine blue O and observed under a light microscope (Nikon OPTI-PHOT). Ultrathin sections were mounted on grids and stained with uranyl acetate followed by lead citrate, and then examined under a transmission electron microscope (Hitachi H-600).

Results and Discussion

1. Light microscopic observation

Firstly, we observed BSC chloroplasts by light microscopy to examine the effects of light intensity on the centrifugal location. Plants used in this study (maize, sorghum, Japanese-millet and Eriachne) are classified into the NADP-ME type C₄ species with the centrifugal arrangement of BSC chloroplasts in the mature leaf (Gutierrez et al., 1974; Prendergast et al., 1987; Sinha and Kellogg, 1996). Mature BSC chloroplasts of the four C₄ plants grown under HL were centripetally oriented (Figs. 1A, D, G and J). However, they showed some differences in BSC chloroplastic orientations between species under LL condition. Although maize maintained the centrifugal arrangement of BSC chloroplasts of Japanese-millet, sorghum and Eriachne was disturbed (Figs. 1E, H and K). BSC chloroplasts of Japanese-millet (Fig. 1E) and sorghum (Fig. 1H) distributed to the adaxial side facing the light source. Such a light-dependent arrangement of BSC chloroplast is a typical response of mesophyll chloroplasts in C₃ plants (Haupt and Scheuerlein, 1990; Trojan and Gabryš, 1996), and this is the first observation of the phototactic movement in C₄ plants. BSC chloroplasts of Eriachne were randomly oriented in the cells under LL (Fig. 1K).

Under LL, MC chloroplasts of Japanese-millet and sorghum were also distributed to the adaxial side like BSC chloroplasts (Fig. 1E and H), while in other two species prominent behaviors of MC chloroplasts were not seen. In the dark, the centrifugal arrangement of BSC etioplasts was observed in maize (Fig. 1C). However, BSC etioplasts of Japanese-millet and sorghum were more or less evenly distributed along the cell walls, while those of Eriachne displayed rather a centripetal orientation (Fig. 1L). From these results, the centrifugal position in maize BSC chloroplasts appears to have a priority over phototactic orientation. By contrast, BSC chloroplasts of other grasses changed their position depending on light intensity and required a higher light intensity for the centrifugal position of BSC chloroplasts.

The centrifugal position of BSC plastids of maize was very rigid even in the dark condition. The rigidity of chloroplast positioning had been observed in finger millet, an NAD- ME enzyme type C₄ plant, in which BSC etioplasts showed centripetal orientation in the dark (Miyake and Yamamoto, 1987). Regulation of motility and positioning of chloroplasts in response to the angle, wavelength and intensity of light is well characterized in C₃ plants and aquatic plants (Haupt and Scheuerlein, 1990; Yatsuhashi, 1996; Kandasamy and Meagher, 1999). Chloroplast movement in response to light inten-
Fig. 1. Light micrographs of transverse sections of leaf blades grown under the HL, LL, and dark conditions. A-C: maize, D-F: Japanese millet, G-I: sorghum, J-L: Eriachne. A, D, G and J, under the HL condition (600 \mu mol m^{-2} s^{-1}); B, E, H and K, under the LL condition (2.5 \mu mol m^{-2} s^{-1}); C, F, I and L, under the darkness. Scale bars = 50 \mu m. BSC, bundle sheath cell; MC, mesophyll cell.

sity optimizes the use of light for photosynthesis. Changes in light quality and intensity are detected by photoreceptors and the photo-signal is transmitted to cytoskeletons (Kandasamy and Meagher, 1999). The organization of actin filaments which interact with chloroplasts is changed and the chloroplasts are oriented properly in relation to the light (Mineyuki et al., 1995). Although actin filaments are also concerned in chloroplast movement (Miyake and Nakamura, 1993), the mechanism of photo-signal transduction is not known in C4 plants. Therefore, the reason for the rigidity observed in maize and finger millet has not been elucidated. However, the present findings indicate that there is a difference in the strictness of centrifugal position of BSC chloroplasts among NADP-ME type C4 grass species and the disposition is differently affected by light inten-
Fig. 2. Light micrographs of transverse sections of leaf blades of Japanese-millet grown in various light intensities. Plants grown for two weeks under 10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (A), 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (B), and 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (C). Scale bars= 50 \( \mu \text{m} \). BSC, bundle sheath cell; MC, mesophyll cell.

Among the four plant species used in this study, Japanese-millet showed the most plastic orientation of BSC chloroplasts with respect to light conditions. Therefore, we examined the distribution of BSC chloroplasts in Japanese-millet by changing the light intensity a little higher than LL (Fig. 2). As compared with the location of BSC chloroplasts under LL \( (2.5 \mu \text{mol m}^{-2} \text{s}^{-1}) \), the number of chloroplasts distributed to the adaxial side decreased as light intensity increased from 10 to 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Figs. 2A to C). These results suggest that Japanese-millet grown under very weak light has a tendency to locate BSC chloroplasts to face the light. When the light intensity increases, Japanese-millet loses the phototactic tendency and instead acquires a tendency of locating the chloroplasts centrifugally. On the contrary, maize seems to possess a strict mechanism for the position-oriented location of BSC chloroplasts, irrespective of light intensity like finger millet (Miyaake and Yamamoto, 1987). Gramineous crops such as maize and sorghum are usually grown in a field with high crop density and their upper leaves make shades. In such circumstances, the lower leaves are forced to photosynthesize under an extremely low light intensity. The present results might also contribute to the study of \( \text{Ci} \) photosynthesis in lower leaves in the field \( \text{Ci} \) crops.

### 2. Electron microscopic observation

The development of grana in BSC chloroplasts of the four plant species grown under HL was more or less suppressed (Fig. 3), although the degree of suppression was different among the species. While the development of grana in BSC chloroplasts of maize and sorghum were strongly suppressed (Fig. 3A and E), considerable development of granal stacking was detected in Japanese-millet and \textit{Eriachne} (Fig. 3C and G). For quantitative analysis, the number of thylakoids per granum in BSC chloroplasts was counted (Table 1). The number of thylakoids per granum was considerably lower in maize and sorghum grown under HL, compared with that of Japanese-millet and \textit{Eriachne}. The grana of \textit{Eriachne} BSC chloroplasts were almost comparable to those of \textit{Japanese-millet}, although it was reported that \textit{Eriachne} possesses developed grana in BSC chloroplasts (Prendergast et al., 1987). Under LL, stacking of thylakoids in BSC chloroplasts of \textit{Japanese-millet} was decreased (Figs. 3D, Table 1). A similar phenomenon was also observed in maize MC chloroplasts as a decreasing granal stacking is caused by extremely weak intensity of light (Nyitrai et al., 1994). Although granal stackings were decreased in some cells, the number of thylakoids per granum in MC chloroplasts was higher than that in BSC chloroplasts regardless of light intensity and species. Therefore, it was concluded that granal development was suppressed in a BSC-specific manner in NADP-ME type species. Granal suppression may be an indispensable or genetically programmed structure in NADP-ME type \( \text{Ci} \) plants, and this structure would not be affected by environmental conditions such as light intensity.

| Plant         | Chloroplast type | No. of thylakoids |
|---------------|-----------------|-------------------|
|               | BSC             | MC                |
| Maize         | 2.4±0.12        | 2.4±0.19          |
| Japanese-millet| 4.4±0.32        | 2.7±0.16          |
| Sorghum       | 2.2±0.03        | 2.1±0.02          |
| \textit{Eriachne} | 3.6±0.05        | 3.3±0.06          |

Japanese-millet, although it was reported that \textit{Eriachne} possesses developed grana in BSC chloroplasts (Prendergast et al., 1987). Under LL, stacking of thylakoids in BSC chloroplasts of \textit{Japanese-millet} was decreased (Figs. 3D, Table 1). A similar phenomenon was also observed in maize MC chloroplasts as a decreasing granal stacking is caused by extremely weak intensity of light (Nyitrai et al., 1994). Although granal stackings were decreased in some cells, the number of thylakoids per granum in MC chloroplasts was higher than that in BSC chloroplasts regardless of light intensity and species. Therefore, it was concluded that granal development was suppressed in a BSC-specific manner in NADP-ME type species. Granal suppression may be an indispensable or genetically programmed structure in NADP-ME type \( \text{Ci} \) plants, and this structure would not be affected by environmental conditions such as light intensity.

The decarboxylation reaction of NADP-ME type species produces NADPH in BSC chloroplasts, which is used in the Calvin cycle. The suppression of granal development in BSC chloroplasts is necessary for this subtype, because highly developed grana of BSC chloroplasts would overproduce NADPH and reduce the efficiency of \( \text{Ci} \) photosynthesis. However, the decarboxylation by NADP-ME produces only 50% NADPH neces-
Fig. 3. Electron micrographs of bundle sheath chloroplasts from transverse sections of leaf blades grown under the HL (600 μmol m⁻² s⁻¹) and LL (2.5 μmol m⁻² s⁻¹) conditions. A and B: maize, C and D: Japanese-millet, E and F: sorghum, G and H: Eriachne. Scale bars = 1 μm. BSC, bundle sheath cell; Gr, grana; MC, mesophyll cell; Mt, mitochondrion; sl, suberized lamella.
sary to reduce glycerate-3-phosphate (G3P) during the Calvin cycle. Agranal chloroplasts have little, or no, ability to produce NADPH by noncyclic electron transport (Ku et al., 1974). The shortage of NADPH to reduce G3P into triose-phosphate (TP) in BSC chloroplasts is considered to be compensated by transport of metabolites between BSC and mesophyll cells (MC) (G3P/TP shuttle) (Stitt and Heldt, 1985; Leegood, 1997; Leegood and Walker, 1999). Thus, G3P/TP shuttle is necessary for C₄ photosynthesis in NADP-ME type, due to the low PSII activity in BSC. Centrifugal position of BSC chloroplasts observed in grasses of NADP-ME type is convenient for operation of this shuttle, and the dependence on this shuttle could also influence the strictness of the position of chloroplasts. Furthermore, the PSI activity of BSC chloroplasts is higher in Digitaria aegyptiaca, an NADP-ME type plant, and with more developed grana in BSC chloroplasts, than the plants with extremely suppressed grana like maize or sorghum (Ku et al., 1974). Therefore, it might be presumed that Japanese-millet and Eriachne, which have somewhat developed grana in BSC chloroplasts, are less dependent on this shuttle than maize and sorghum. However, the results of granal development of BSC chloroplasts were not consistent with their localization, since only maize had a strict centrifugal position of BSC chloroplasts under all light conditions while remarkable suppression of granal development in BSC chloroplasts were observed in both maize and sorghum.

These findings suggest that the strictness of the centrifugal location of BSC chloroplasts is unrelated to the degree of their granal development, and the structure-function relationships in C₄ plants might be different among species within the same decarboxylation group. Further work such as measurements of metabolite transports between BSC and MC chloroplasts is needed to clarify the mechanism for the specific location of BSC chloroplasts, which will also help understand the significance of the location of BSC chloroplasts in C₄ photosynthesis.

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