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AUTHOR(S):

Maai, Eri; Nishimura, Kazusa; Takisawa, Rihito; Nakazaki, Tetsuya

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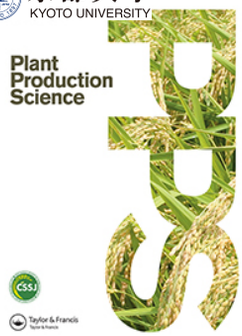

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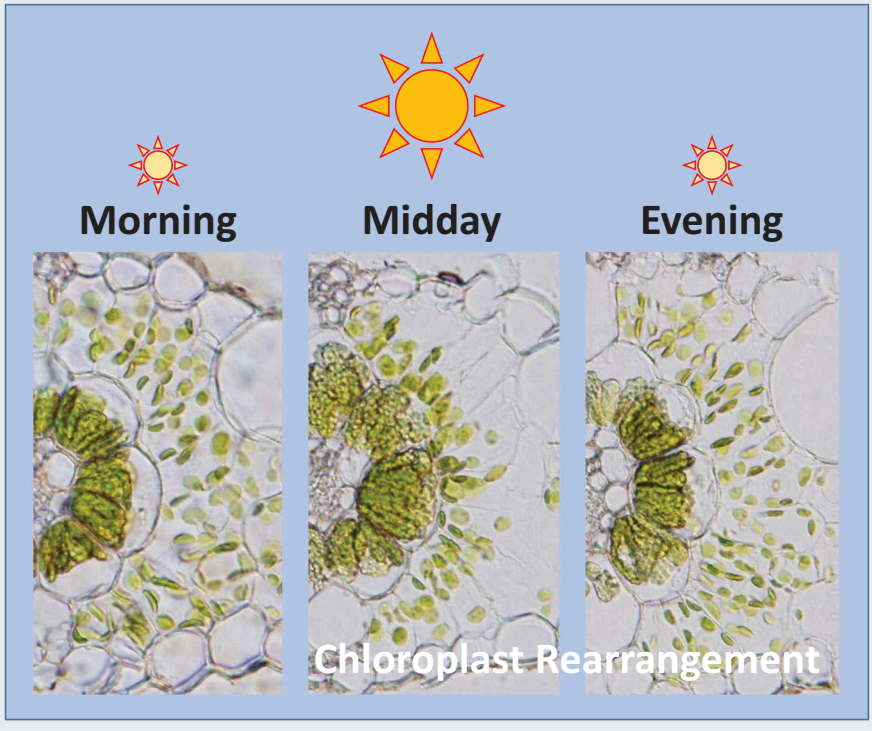
Diurnal changes in chloroplast positioning and photosynthetic traits of C_4 grass finger millet

Eri Maai, Kazusa Nishimura, Rihito Takisawa and Tetsuya Nakazaki

Graduate School of Agriculture, Kyoto University, Kyoto, Japan

ABSTRACT

Mesophyll (M) chloroplasts in finger millet are known to aggregate to the bundle sheath side when leaves are constantly irradiated with extremely high-intensity light. This aggregative movement of M chloroplasts is also observed in natural environment, but whether a natural light regime is effective in inducing the response remains unclear. Abscisic acid is reported to trigger not only the aggregative movement but also stomatal closure, but photosynthetic responses accompanying the aggregative movement also remain unknown.We investigated changes in chloroplast positioning and photosynthetic traits under diurnal patterns of light, mimicking the natural light environment. M chloroplasts showed the aggregative movement with increasing light intensity whether it frequently fluctuated or not, and kept their aggregative positions in the midday. With decreasing light intensity, M chloroplasts returned to the random position in the evening. These results suggest that M chloroplasts often rearrange their intracellular positions during the daytime and that the chloroplast aggregative movement can be induced by a natural regime of light. The chloroplast aggregative movement was observed with increasing stomatal conductance, suggesting that stomatal closure is not crucial to trigger the chloroplast response.



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Introduction

In many plants, the intracellular location of chloroplasts is regulated by light intensity and quality (Haupt, 1973; Inoue & Shibata, 1974; Trojan & Gabryś, 1996; Wada et al., 2003). Chloroplast photorelocation movement has been well-studied and characterized in a C_3 plant, *Arabidopsis*

CONTACT Eri Maai maai.eri.6r@kyoto-u.ac.jp
Supplemental data for this article can be accessed here.

thaliana, with two distinct phenomena: accumulation movement, in which chloroplasts move toward the light under low or moderate light intensity, and avoidance movement, in which chloroplasts move away from strong light (Kasahara et al., 2002; Sakai et al., 2001; Takagi, 2003; Wada et al., 2003). The former is an essential response to optimize light capture under low light conditions, while the latter is effective in minimizing photodamage under high light intensity. Accordingly, these two responses are considered to be beneficial to the safe and efficient utilization of light. In contrast to studies of chloroplast photorelocation movement in algae and C_3 plants, there have been only a few studies of this phenomenon in C_4 plants. In C_4 plants, photosynthetic cells are structurally and functionally differentiated into bundle sheath (BS) cells and mesophyll (M) cells. BS chloroplasts are classically located either centripetally or centrifugally to the vascular tissue, whereas M chloroplasts are randomly distributed along the cell walls (Dengler & Nelson, 1999). It has previously been reported that M chloroplasts aggregately move to the BS side in response to exposure to high-intensity light, whereas BS chloroplasts do not change their intracellular positions (Yamada et al., 2009). Although the aggregation of chloroplasts is observed in crassulacean acid metabolism (CAM) plants including *Opuntia streptacantha* (Delgado-Sanchez et al., 2013) and *Karanchoë fedtschenkoi* (Kondo et al., 2004) and C_3-C_4 intermediate plants including *Halophila stipulacea* (Drew, 1979), those chloroplasts densely clump in the center, or sometimes two areas of the cytoplasm under light (Sharon & Beer, 2008). These chloroplast arrangements are called chloroplast clumping or chloroplast dispersal and are different phenomena in the direction of the movement from the response in C_4 plants whose M chloroplasts move toward the BS side. The aggregative movement of M chloroplasts has been identified only in C_4 plants and it also differs from the high light-induced avoidance movement of chloroplasts in C_3 plants in requiring longer exposure and a higher light intensity to cause the response. For instance, continuous white light at more than 3000 μmol quanta m^{-2} s^{-1} for several hours is needed to elicit the aggregative movement of M chloroplasts in finger millet (Yamada et al., 2009), whereas exposure to 500 μmol m^{-2} s^{-1} can cause the avoidance movement in *Arabidopsis thaliana* within an hour (Kasahara et al., 2002). Previous studies reported that the aggregative movement of M chloroplasts can also be induced by drought (Lal & Edwards, 1996; Yamada et al., 2009). In the meantime, the response was detected in field-grown finger millet under well-watered conditions as well (Yamada et al., 2009). The authors pointed out that the leaf surface of the field-grown plant was irradiated with 1800 μmol m^{-2} s^{-1} of sunlight, which was not high enough to induce the chloroplast aggregative movement of well-watered plants in the laboratory. The factor(s) that induced the aggregative movement of M chloroplasts under well-watered field conditions remains unknown. It should be noted that laboratory experiments in Yamada et al. (2009) were conducted under continuous light conditions. However, irradiation in natural environment varies and often fluctuates over the course of a day due to cloud cover or overshadowing canopy cover, and plants rarely receive constant levels of light (Pearcy, 1990). Therefore, in order to interpret the effect of light on the chloroplast aggregative movement found in natural environment, chloroplast positioning under a natural light regime in the absence of drought required to be investigated.

The aggregative movement of M chloroplasts in response to constant high-intensity light is observed not only in finger millet but also in other C_4 plant, maize. Yamada et al. (2009) mentioned that maize chloroplasts preferentially showed the avoidance movement compared to those of finger millet which preferentially showed the aggregative movement, suggesting that C_4 plants conserve the mechanism of the chloroplast aggregative movement but chloroplast behavior varies with C_4 plant species. In general, C_4 plants are classified by their major decarboxylation enzymes (i.e. NADP-malic enzyme (ME), NAD-ME, and phospho*enol*pyruvate carboxylase) (Hatch, 1987). Maize is an NADP-ME type C_4 plant which possesses a suberized lamella in BS cell walls, whereas finger millet is an NAD-ME type C_4 plant which does not possess the one (Hattersley & Browning, 1981; Ohsugi et al., 1988). A role of the suberized lamella is thought to prevent CO_2 leakage from BS cells. Therefore, it has been supposed that M chloroplasts and cytosol in finger millet, which does not have a suberized lamella, preferentially aggregate towards the BS side to refix CO_2 released from BS cells more efficiently (Yamada et al., 2009). In addition, they have suggested that the aggregative movement of M chloroplasts to the BS side shortens the diffusion pathway of photosynthetic metabolites between M and BS cells and may contribute to the efficiency of C_4 photosynthesis under stress conditions. However, the physiological significance of the aggregative movement of C_4 chloroplasts remains unknown. If the aggregative movement of M chloroplasts relates to photosynthetic performance of leaves under light stress conditions, changes in chloroplast positioning along with changes in photosynthetic traits would be worth consideration. Intriguingly, our previous study had found that exogenous abscisic acid (ABA) promotes M chloroplasts to exhibit aggregative movement in finger millet (Maai et al., 2011). Since

ABA plays an important role in plant responses, especially in stomatal closure (Mittelheuser & Vansteveninok, 1969; Zhang et al., 2006), it is assumed that stomatal closure triggers the aggregative movement of M chloroplasts. Recently, we investigated the positioning of chloroplasts and photosynthetic responses using an NADP-ME type C_4 plant, sorghum (Maai et al., in press). The study revealed the relation between the chloroplast avoidance movement and midday depression of photosynthesis. However, sorghum chloroplasts showed the apparent avoidance movement rather than the aggregative movement as those of maize, and thus we still have little information about the photosynthetic response accompanying the chloroplast aggregative movement.

The aim of this study is to clarify the involvement of a natural regime of light in the aggregative movement of M chloroplasts in a C_4 plant, finger millet. Another aim is to investigate changes in photosynthetic traits along with changes in chloroplast positioning during irradiation.

Materials and methods

Plant material and growth condition

Finger millet (*Eleusine coracana* L. Gaertn., 'Yukijirushi', Snow Brand Seed Co., Ltd, Japan) was grown in 8 cm diameter × 8 cm tall plastic pots filled with a mixture of vermiculite and a commercial substrate (Ikubyo baido, Takii and Co., Japan) (1:1 v/v) in a growth chamber with a 14-h/10-h 28/20°C day/night cycle (light intensity of 500 µmol quanta m^{-2} s^{-1}) for 3–4 weeks. Plants were irrigated twice a day and fertilized with a commercial nutrient solution containing 6–10–5 (N–P–K) (Hyponex, Hyponex Japan Co., Osaka, Japan) periodically. The middle regions of the uppermost fully elongated leaves were used for experiments. To exclude the effect of light exposure before the experiments, plants were kept in darkness for at least 6 h prior to experiments.

Treatments with a diurnal pattern of light, mimicking a natural environment

The leaf was placed in a 6 cm^2 chamber attached to a portable gas-exchange measurement system (LI-6400, LI-COR, Inc., NE, USA), before being exposed to the diurnal light regime for up to 12 h. Light was provided via an LED lamp (6400-02B, LI-COR) which emits red (peak wavelength 665 nm) and blue light (peak wavelength 470 nm). The irradiation light regime 1 was reproduced from a natural light regime recorded at the Experimental Farm of Kyoto University (34°44′N, 135°50′E) from 06:00 h to 18:00 h on a fair day in early August, with a modification as follows. Because the LED lamp can produce a light intensity of up to 2000 µmol m^{-2} s^{-1}, recorded values exceeding a maximum output of the LED lamp (2002 µmol m^{-2} s^{-1} at 10:53 h, 2036 µmol m^{-2} s^{-1} at 11:44 h and 2019 µmol m^{-2} s^{-1} at 11:45 h) were each adjusted to 2000 µmol m^{-2} s^{-1}. Similarly, the irradiation light regime 2 was reproduced from a natural light regime recorded at the same site from 06:00 h to 18:00 h on a clear day in late July. The lengths of daytime were almost equal between in late July and in early August. The average irradiance during the 12 h irradiation regime was 988 µmol m^{-2} s^{-1} in regime 1 and 1227 µmol m^{-2} s^{-1} in regime 2, respectively (Figure 1).

To examine the effect of the circadian rhythm on chloroplast rearrangement, leaves were kept in darkness at 28°C for 12 h instead of treating with light as described above.

Observation of chloroplast arrangements

At each of 0, 4, 6, 8, and 12 h of light treatments, small leaf segments were excised and incubated with fixation buffer [3% (v/v) glutaraldehyde and 50 mM Na-phosphate, pH 6.8] at 4°C overnight. The fixed leaf segments were embedded into Super Cryo Embedding Medium (SCEM, Section Lab, Japan) such that cutting plane for the region of interest is parallel with the bottom of the cryomold, and then frozen in hexane-dry ice. The surface of the embedded blocks was tightly covered with an adhesive film (Cryofilm Type 2 C(10), Section Lab) and then carefully sliced at 10–15 µm thickness with a cryostat (CM3050 S, Leica BioSystems, Germany) at −20°C, according to the Kawamoto protocol (Kawamoto, 2003). Transverse sections were observed with a light microscope (BX51, Olympus Co., Tokyo, Japan) equipped with a charge-couple device camera (DP70, Olympus).

We also observed transverse sections of the dark treated leaves in the same way.

Quantification of chloroplast arrangements

To quantify the extent of the aggregative movement of M chloroplasts, nine vascular Kranz units were sampled from each of three biological replicates, each replicate containing leaf tissues from three individually treated plants; all M cells, excluding any which were unclear, were sampled for the purpose of quantification. For each side of a leaf, the coordinates of each chloroplast, the ends of the M cell walls in contact with a neighboring BS cell, and the innermost point from the BS-side M cell wall in the digital image of the transverse section were determined using software (Katikati Counter; GT Soft, Tokyo, Japan). We calculated the relative distance from the BS-side cell walls to each chloroplast in adaxial and

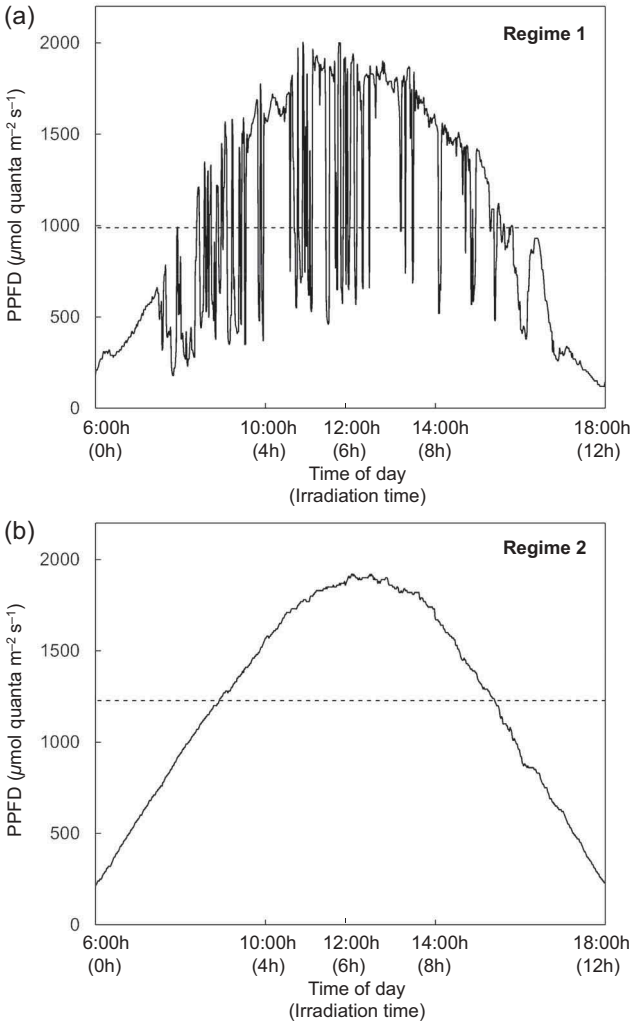

Figure 1. Light conditions during the experiments. The light regimes for light treatments were based on the actual incident light [Photosynthetic Photon Flux Density (PPFD) in μmol quanta m^{-2} s^{-1}] measured at the experimental site on a fair day (regime 1; a) and on a clear day (regime 2; b). The data set of PPFD from 06:00 h to 18:00 h in each regime was used for the 12 h light irradiation experiments. The average intensity of the irradiation light was 988 μmol m^{-2} s^{-1} in regime 1 and 1227 μmol m^{-2} s^{-1} in regime 2, as each indicated by the dotted line.

abaxial M cells, and then determined the index of aggregative arrangement (%) as described previously (Maai et al., 2011). The average values of this index on each side of a leaf were subjected to statistical analyses. This index value indicates the extent of distribution of M chloroplasts toward the BS side. As M chloroplasts aggregate to the BS side, the index value increases and approaches 100%.

Leaf gas-exchange

During the light treatment, net photosynthetic rate (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}) and intracellular CO_2 concentration (Ci, μmol mol^{-1}) were analyzed at 1 min intervals. Measurements were performed at 28°C with a relative humidity of 50%–70% and an ambient CO_2 concentration of 350–400 μmol mol^{-1}. A constant flow rate was set at 500 μmol s^{-1}.

Chlorophyll fluorescence

To determine the maximum quantum efficiency of photosystem (PS) II, chlorophyll fluorescence was measured using a hand-held chlorophyll fluorometer (FluorPen FP110/S, Photon Systems Instruments, Drásov, Czech Republic). Plants were kept in darkness for at least 6 h prior to the light treatment, and then, dark-adapted the maximum quantum efficiency of PS II (termed as *Fv/Fm*) was measured as a value at t = 0 h. After each of 4, 6, 8, and 12 h light treatment, plants were kept in the dark for 5 min and then light-adapted maximum quantum efficiency of PS II (termed as *Fv′/Fm′*) were measured. This is because positions of chloroplasts may be rearranged during long-term dark adaptation for more than 30 min to obtain *Fv/Fm* values. Chloroplast positions after short-term dark adaptation would be closer to those just after the light treatment. Thus, we measured *Fv′/Fm′* values to assess the maximum quantum efficiency of PS II in a specific chloroplast arrangement after the light treatments. In order to avoid the influence of a temporal dark treatment and of high-intensity exciting light on the position of the chloroplasts, the light treatments for measuring chlorophyll fluorescence and observing chloroplast arrangements were conducted independently.

Statistical analysis

In each experimental light regime, pearson correction between P_n and g_s was analyzed. On each side of a leaf, the statistical significance of differences in the index values of aggregative arrangement among each time point was assessed by Tukey's multiple comparison test at $P < 0.05$. The statistical significance of differences in the maximum efficiency of PS II was analyzed by ANOVA with Scheffe's test at $P < 0.05$. Calculations were performed on three independent biological replicates. Percentage data were subjected to arcsine transformation prior to statistical analysis.

Results

Changes in the position of chloroplasts in response to a natural regime of light

We investigated the diurnal responses of chloroplast position under two different natural light regimes, mimicking

typical natural light environments from 06:00 h (dawn) to 18:00 h (sunset), using an LED lamp. The experimental light regime 1 reproduced a natural light environment on a fair day with frequent fluctuations of light intensity (Figure 1(a)). On the contrary, the light regime 2 reproduced that on a clear day with less fluctuations of light intensity (Figure 1(b)). Finger millet is classified into the NAD- ME type C_4 species with the centripetal position of BS chloroplasts (Miyake & Yamamoto, 1987). During both light treatments, BS chloroplasts kept their centripetal positions (Figure 2). By contrast, we observed a diurnal pattern of M chloroplast positioning in response to light. Before the light treatment, M chloroplasts were randomly located along the plasma membranes (Figure 2(a,b)). In regime 1, M chloroplasts aggregated to the BS side after 4 h irradiation (Figure 2(c)). During the period of 4–8 h irradiation, leaves were intermittently exposed to high intensity light in excess of 1500 µmol m^{-2} s^{-1}, and the apparent aggregative movement of M chloroplasts at both leaf sides was observed after 6 h irradiation (Figure 2(e)). The extent of chloroplast aggregation in abaxial M cells seemed to be relatively weak. The aggregative position of M chloroplasts was maintained even after 8 h irradiation (Figure 2(g)). After 12 h irradiation, at which point the light intensity decreased, most M chloroplasts returned to random positions along the plasma membranes (Figure 2(i)). In regime 2, the aggregative movement of M chloroplasts was observed in a similar fashion (Figure 2(b,d,f,h,j)). The extent of chloroplast aggregation seemed to be enhanced during the period of 4–6 h irradiation while increasing light intensity with less fluctuations. These results from tissue observation were confirmed by quantitative analyses of the index values of aggregative arrangement of M chloroplasts. In regime 1, the index values at both leaf sides increased significantly after 4 h of irradiation, and there were no significant differences among those during the period of 4–8 h irradiation (Figure 3(a)). At both leaf sides, the decreased index values were detected after 12 h irradiation compared to those after 6 h irradiation. The index values (%) at the adaxial side were 14.5, 11.8, 14.4, and 9.8 higher than those at the abaxial side after 4 h, 6 h, 8 h, and 12 h of irradiation, respectively. In regime 2, the index values at both leaf sides also increased significantly after 4 h irradiation but continued to increase during the period of 4–6 h irradiation (Figure 3(b)). At both leaf sides, the index values decreased significantly after 12 h irradiation compared to those after 8 h irradiation. The index values (%) at the adaxial side were 5.8, 8.7, 5.9, and 9.8 higher than those at the abaxial side after 4 h, 6 h, 8 h, and 12 h of irradiation, respectively. By contrast, no chloroplast rearrangement was observed in the dark (Suppl. data 1).

Diurnal responses of gas-exchange parameters under a natural light regime

To investigate the effect of the light treatments on photosynthetic traits, we measured gas-exchange parameters every minute throughout the light treatments. In regime 1, both P_n and g_s responded rapidly to sudden fluctuation in light intensity (Figure 4(a,c)). Each of them reached a maximum during the period 4–6 h of irradiation and decreased thereafter. There were strong positive correlations between P_n and g_s (r^2 = 0.9737) (data not shown). *Ci* values decreased at the beginning of irradiation. They also fluctuated along with light fluctuations but broadly kept constant at 100 µmol mol^{-1} or thereabout (Figure 4(e)). By contrast, in regime 2, P_n and g_s increased smoothly (Figure 4(b,d)). They reached maxima around 4 h after irradiation and decreased thereafter. g_s was positively correlated to P_n (r^2 = 0.9310) (data not shown). *Ci* values decreased with increasing P_n at the beginning of irradiation but kept relatively constant at around 100 µmol mol^{-1} thereafter (Figure 4(f)). This is consistent with the findings of *Ci* with a tendency to remain nearly constant (Hsiao, 1993; Wong et al., 1979).

As shown in Figure 5, we assessed how P_n was affected by various light intensities during the treatment period. In either regime of the light treatment, P_n values increased with increasing light intensity. The average value of P_n was 17.7% and 19.8% higher during the period 0–6 h of the light treatment than during 6–12 h in regime 1 and regime 2, respectively. The light environment in the former time-period corresponded to the time from 06:00 to 12:00, whereas the latter corresponded to that from 12:00 to 18:00 (Figure 1). In other words, finger millet leaves showed a decrease in photosynthetic performance in the afternoon.

Photoinhibition under a natural light regime

The effect of the light treatments on chlorophyll fluorescence of leaves is shown in Figure 6. In either regime of the light treatment, the values of the maximum efficiency of PS II decreased significantly at each time point from 4 h of irradiation onwards. The values in regime 1 decreased by 10.5, 20.6, 22.4, and 19.8% after 4, 6, 8, and 12 h of irradiation compared with that before the treatment, respectively. On the contrary, the values in regime 2 decreased deeply by 26.3, 44.1, 46.6, and 26.3% after 4, 6, 8 and 12 h of irradiation compared with that before the treatment, respectively. As irradiance decreased from 8 h through 12 h of the treatment, the value in regime 2 increased even though it was still lower than that before the treatment.

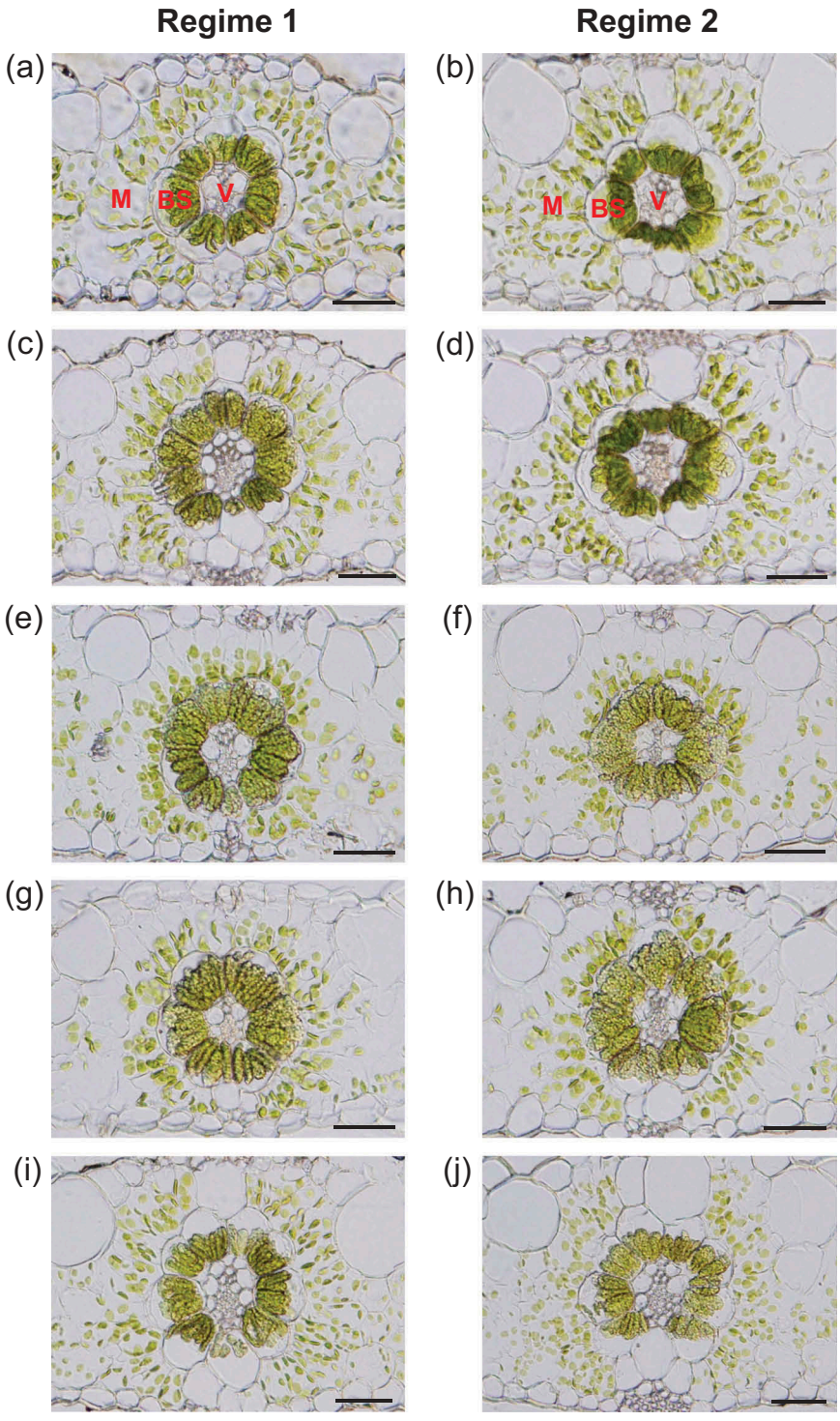

Figure 2. Diurnal changes in the intracellular arrangement of chloroplasts under light regime 1 (a, c, e, g, i) and light regime 2 (b, d, f, h, j) (see Figure 1). Transverse sections were observed with a light microscope before (a, b), 4 h (c, d), 6 h (e, f), 8 h (g, h), and 12 h (i, j) after the start of light irradiation. The adaxial side of each leaf blade (upper side in the photogragh) was irradiated. Scale bars = 50 µm. BS, bundle sheath cell; M, mesophyll cell; V, vascular bundle.

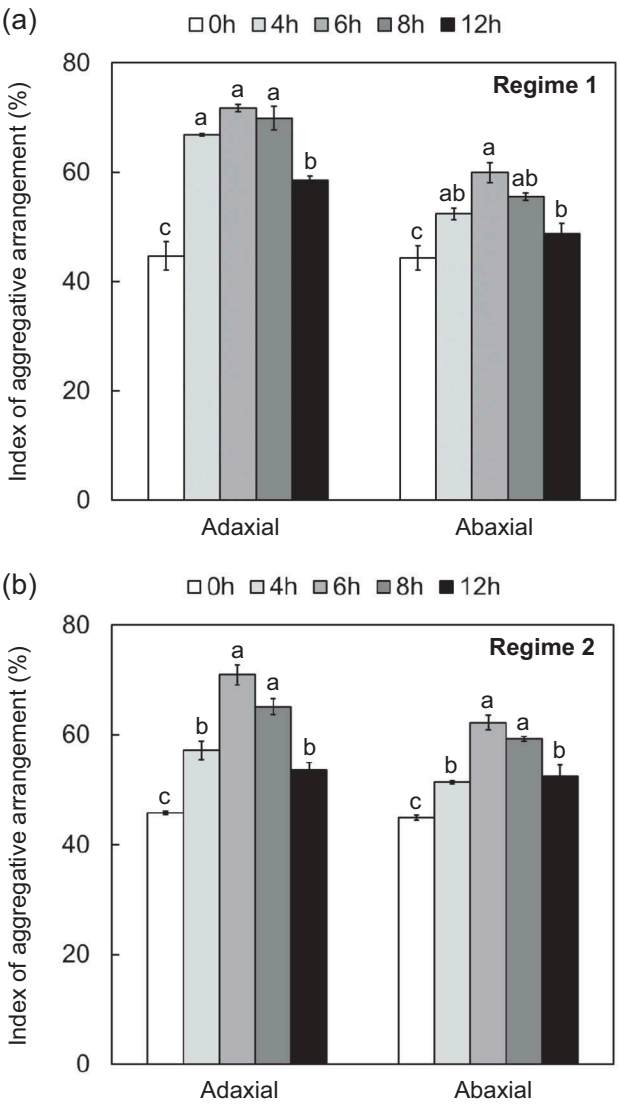

Figure 3. Effects of the light treatment on the index of aggregative arrangement of mesophyll chloroplasts in adaxial and abaxial M cells in regime 1 (a) and in regime 2 (b). The results are the mean values (± SE) of three biological replicates, each containing leaf tissues from three individually treated plants (n = 35–187 chloroplasts). At each leaf side, different letters above the bars indicate a significant difference at $P < 0.05$ among each time point of irradiation (Tukey's test).

Discussion

Effect of a natural regime of light on the chloroplast aggregative movement

To clarify the involvement of a natural regime of light in triggering the aggregative movement of M chloroplasts under well-watered conditions, we investigated changes in chloroplast positioning during two different natural light regimes with frequent or less fluctuations of light intensity (Figure 1). In many studies, chloroplast movement is analyzed by measuring changes in leaf transmittance or absorbance (Wada et al., 2003). An increase in leaf transmittance indicates the chloroplast avoidance movement and a decrease indicates the accumulation movement (Trojan & Gabryś, 1996; Walczak & Gabryś, 1980). Unfavorably, leaf transmittance is also increased by the chloroplast aggregative movement (Yamada et al., 2009), and it would not be easy to distinguish the avoidance movement from the aggregative movement by changes in leaf transmittance. For this reason, leaf transmittance was not investigated to detect the chloroplast movement in the present study. From microscope observation, we found that M chloroplasts in well-watered finger millet showed the aggregative movement with increases in light intensity whether it frequently fluctuated or not (Figure 2(c–h)). Because this chloroplast response was not observed in the dark (Suppl. data 1), it is suggested that the chloroplast aggregative movement is not triggered by the circadian rhythm. Furthermore, our data indicate that a natural light regime is effective in inducing the aggregative movement of M chloroplasts, and besides, this theory can explain the findings of Yamada et al. (2009) that M chloroplasts showed the aggregative movement under well-watered field conditions. The index of the aggregative arrangement in regime 1 greatly increased during the period 0–4 h of irradiation, whereas that in regime 2 increased stepwisely along with an increase in light intensity during the period 0–6 h of irradiation (Figure 3). This suggests that the speed and the extent of the aggregative movement of M chloroplasts may vary with light conditions.

To the best of our knowledge, our study is the first report on chloroplast rearrangement from the aggregative position to the random position in response to light. A recent study has reported that the intracellular disposition of finger millet M chloroplasts was disrupted by centrifugal forces but M chloroplasts returned to random positions within 2 h in the dark (Kobayashi et al., 2009). Another study has revealed that M chloroplasts of field-grown finger millet, which had arranged in the aggregative positions in the daytime, rearranged in random positions at night (Yamada et al., 2009).It has not been clarified whether M chloroplasts in finger millet rearrange their intracellular positions to random positions in the light. In this study, we found that M chloroplasts moved back to random positions during the period of 8–12 h irradiation even though their aggregative position was not fully recovered in both regimes of light treatments (Figure 2(i,j); Figure 3). Our data suggest that M chloroplasts in finger millet can often rearrange their intracellular positions in response to the light environment during the daytime.

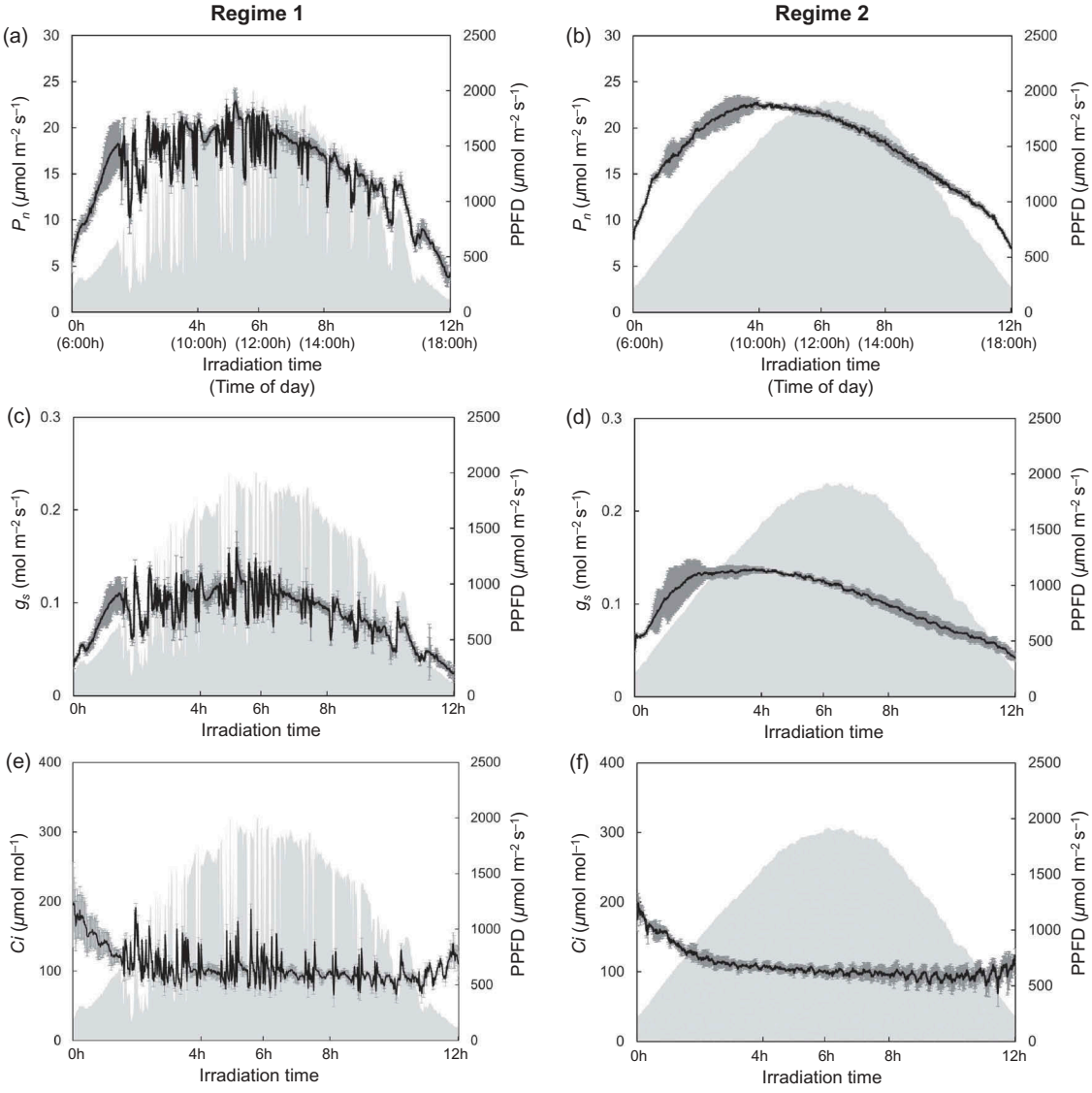

Figure 4. Diurnal changes in photosynthetic traits under light regime 1 (a, c, e) and light regime 2 (b, d, f). The net photosynthetic rate (P_n) (a, b), stomatal conductance (g_s) (c, d), and intracellular CO_2 concentration (Ci) (e, f) were measured at 1 min intervals during each regime of the light treatment. The measurements were performed at 28°C, a relative humidity of 50%–70% and an ambient CO_2 concentration of 350–400 μmol mol^{-1}. Each data point represents the mean values (± SE) of three biological replicates. Gray shaded areas represent irradiation light regimes for the measurements.

Chloroplast photorelocation movement is regarded to be one of the adaptations for dealing with changing light environment (Li et al., 2009; Wada et al., 2003), and its response varies with environment and species (Higa & Wada, 2016; Park et al., 1996; Trojan & Gabryś, 1996). Recently, we observed the avoidance movement of M chloroplasts in sorghum leaves in response to a natural regime of light, which was similar to regime 1 in the present study (Maai et al., in press). By contrast, M chloroplasts of finger millet showed the aggregative movement under such conditions. This means that the chloroplast response differs from C_4 species, as reported in Yamada et al. (2009). Intriguingly, finger millet M chloroplasts at the adaxial side showed the aggregative movement more strongly than those at the abaxial side after 4 h, 6 h, and 8 h of irradiation in both regimes of light treatments (Figure 2(c–h); Figure 3). C_4 plants have complex leaf structures, composed of M cells and BS cells. BS cells generally contain large chloroplasts located either centripetally or centrifugally to the vascular tissue. Bellasio and Lundgren (2016) suggested that pigmented BS extensions result in decreased light penetration through BS cells. Decreased intensities of light leads to a decrease in the extent of the aggregative movement of M chloroplasts in finger millet (Maai et al., 2011). Because the adaxial side of each leaf was illuminated, it

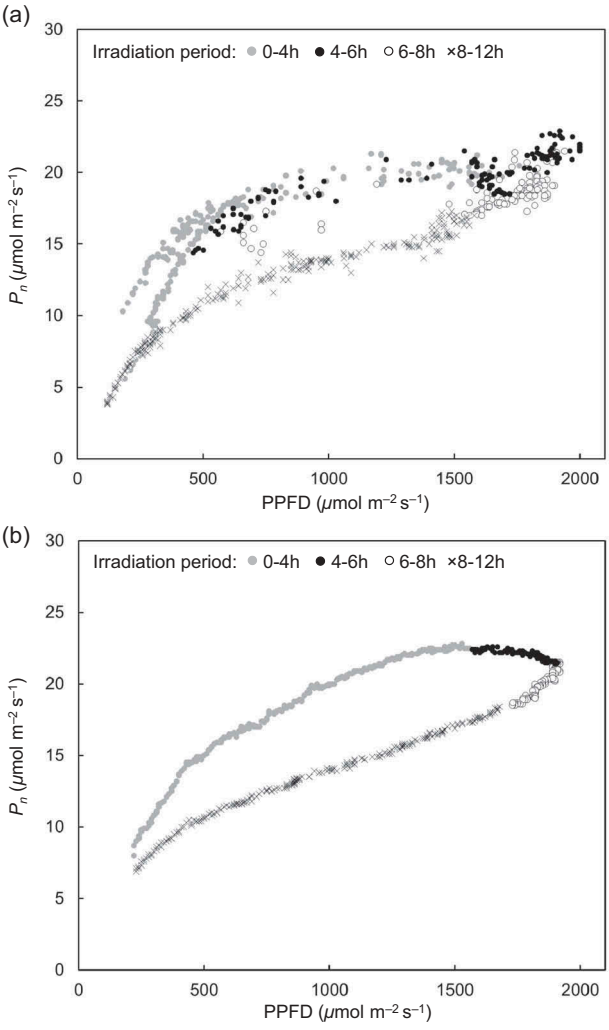

Figure 5. Relationships between the net photosynthetic rate and light intensity under light regime 1 (a) and light regime 2 (b). Values are the mean; $n = 3$. Values measured during 0–4 h, 4–6 h, 6–8 h and 8–12 h of light treatment are represented by gray circles, black circles, white circles and crosses, respectively.

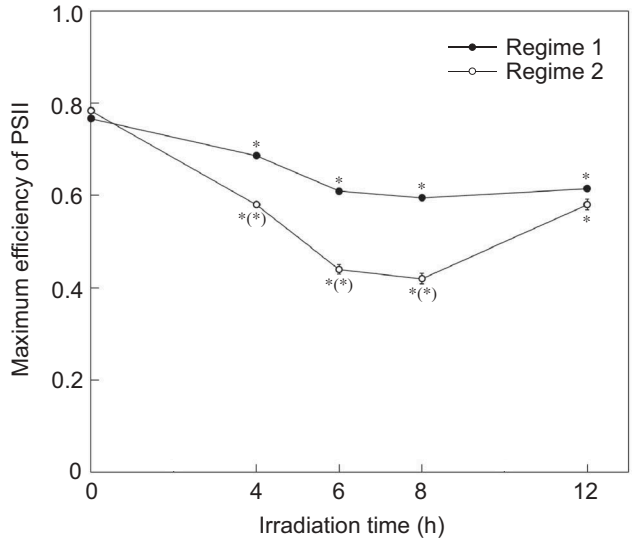

Figure 6. Effects of light treatments on the maximum efficiency of photosystem (PS) II. Values were measured before and after 4, 6, 8, and 12 h of the light treatments. Asterisks indicate statistically significant differences compared with t = 0 h in each light regime according to ANOVA with Scheffe's test ($P < 0.05$). Comparisons between the figures at each time point are shown in parentheses. The results are the means ± SE from three independent experiments.

would be possible that weakened irradiance at the abaxial side decreased the extent of M chloroplast movements compared to those at the adaxial side.

Response of photosynthesis and its relation to chloroplast arrangement

Our study is the first report that shows changes in photosynthetic traits accompanying the chloroplast aggregative movement. In both regimes, P_n and g_s increased with increasing light intensity (Figure 4(a–d)) and there were positive correlations between these two photosynthetic parameters. These results are in accordance with findings in several C_4 species (Fernandez et al., 2015; Tsutsumi et al., 2017; Wong et al., 1985; Yabiku & Ueno, 2017). In both regimes, P_n values measured during 6–12 h of light treatments were almost always lower than those during 0–6 h (Figure 5). This depression of photosynthesis in the afternoon is commonly observed in many plants (Hirasawa & Hsiao, 1999; Hirasawa et al., 1992; Jifon & Syvertsen, 2003) and stomatal closure is considered to be one of the major factor responsible for this phenomenon (Hirasawa et al., 1989; Iio et al., 2004; Muraoka et al., 1997). This would be applied to the results of our study. Since the decrease in P_n was in accordance with the decrease in g_s, it is thought that the decreased P_n resulted from stomatal limitation.

ABA is a major factor inducing stomatal closure (Mittelheuser & Vansteveninok, 1969; Zhang et al., 2006), which is represented by decreased g_s. Another role of ABA is thought to trigger the aggregative movement of M chloroplasts (Maai et al., 2011; Yamada et al., 2009). Therefore, we assumed that the chloroplast aggregative movement accompanies a decrease in g_s. Nevertheless, the chloroplast aggregative movement was observed during 0–4 h of light treatments when both P_n and g_s increased with increasing light intensity (Figure 2(a–d); Figure 4(a–d)). During 0–4 h of light treatments, we found little temporal decreases in g_s values in regime 2 (Figure 4(d)) whereas there were frequent temporal decreases in those in regime 1 (Figure 4(c)). These results suggest that stomatal closure is not crucial to trigger the chloroplast aggregative

movement. Stomatal opening and closure are regulated by a complex network of signaling pathways, which includes the ABA signaling pathway (Huang et al., 2008; Nemhauser et al., 2006). Thus, it is possible that the action of stomata can be changed alternately regardless of the presence or absence of ABA. Although the changes in the concentration of endogenous ABA were not analyzed in the present study, the occurrence of the chloroplast aggregative movement indicates a possibility that there was a sufficient concentration of endogenous ABA to induce the chloroplast response in leaves. On the other hand, it is also possible that the chloroplast aggregative movement is induced via the pathway independently of ABA, in which light may be involved.

During both regimes of light treatments, *Ci* values decreased at the beginning of irradiation while P_n values greatly increased (Figure 4(a,b,e,f)).Because photosynthesis consumes intracellular CO_2, it is suggested that a decrease in *Ci* is derived from the start of photosynthesis. During the period 0–4 h of irradiation, when *Ci* values decreased, M chloroplasts began to aggregate to the BS side (Figure 2(c,d)). These results can be explained by the hypothesis that M chloroplasts and cytosol aggregate towards the BS side to retrieve CO_2 released from BS cells more efficiently (Yamada et al., 2009). It is intriguing that the aggregative arrangement of M chloroplasts was almost completed after 4 h irradiation in regime 1 whereas it was stepwisely completed until 6 h irradiation in regime 2 (Figure 3), suggesting that the speed and the extent of the aggregative movement of M chloroplasts may vary with light conditions. C_4 photosynthesis depends on spatial separation of photosynthetic reactions: light-dependent carbon fixation in M cells and carbon assimilation via the Calvin cycle in BS cells (Hatch, 1992). The efficiency of C_4 photosynthesis depends on the coordination of these two photosynthetic reactions. To maintain C_4 photosynthesis, reduced carbon must be transported from BS cells back into M cells to replace the carbon transported by the original C_4 carbonic acid. In a recent study, it is proposed that this coordination is impacted by fluctuating light (Slattery et al., 2018). For example, a high to low light transition results in a transient overpumping of CO_2 into BS cells and subsequent CO_2 leakage out of BS cells (Cousins et al., 2008; Pengelly et al., 2010). By contrast, a low to high light transition results in higher rates of Rubisco oxygenation, incurring increased photorespiration (Von Caemmerer & Furbank, 2003). Yamada et al. (2009) proposed that the aggregative movement of M chloroplasts shortens the diffusion pathway of photosynthetic metabolites between M and BS cells, which may contribute to the efficient operation of C_4 photosynthesis. From these findings together, it is likely that a fluctuating light regime allowed M chloroplasts to aggregate to the BS side more rapidly to deal with the imbalanced C_4 photosynthetic cycle between M and BS cells.

In both regimes, the maximum efficiency of PS II decreased significantly in the daytime (Figure 6). This indicates that leaves were exposed to excessive light energy, sufficient to cause photoinhibition. It is known that chloroplasts in many terrestrial plants escape from strong light to reduce photodamage (Kasahara et al., 2002; Wada et al., 2003). High light-induced aggregative movement of C_4-M chloroplasts increases leaf transmittance as is the case with the avoidance movement (Inoue & Shibata, 1973; Walczak & Gabryś, 1980; Yamada et al., 2009). Therefore, it is thought that there was the successive need to avoid exposure to excessive light energy and M chloroplasts kept their aggregative positions in the midday. It is worth noting that the maximum efficiency of PS II under fluctuating light regime decreased more moderately than under less-fluctuating light regime during the period 0–6 h of irradiation (Figure 6). Possible explanations for this difference in the maximum efficiency of PS II include the difference in the amount of light received during both conditions. Importantly, we found that the speed and the extent of the aggregative movement of M chloroplasts may vary with light conditions (Figure 2; Figure 3). Therefore, it is also possible that the rapid aggregation of M chloroplasts resulted in mitigating the extent of photoinhibition under fluctuating light regime. These possibilities should be analyzed in future research. In *Arabidopsis thaliana, phot2* mutant line defective in the chloroplast avoidance movement showed constitutive accumulation movement under fluctuating light conditions and showed stronger photoinhibition than wild-type plants (Gotoh et al., 2018). The authors addressed that chloroplast positioning under fluctuating light conditions was essential for the photosynthetic apparatus especially in escaping from the sudden strong light-induced photoinhibition. Whether the aggregative movement in finger millet is more effective in mitigating photoinhibition than the avoidance movement remains an intriguing issue. Importantly, finger millet chloroplasts showed the aggregative movement in response to a diurnal regime of light, although the plant possesses both mechanisms of the chloroplast movement; the avoidance and the aggregative movement (Maai et al., 2011). In addition, we found a tendency that M chloroplasts completed aggregating to the BS side within a relatively short period under fluctuating light conditions, where C_4 photosynthesis was prone to get imbalanced, compared to less-

fluctuating light conditions (Figure 2; Figure 3). If M chloroplasts in finger millet need only to escape from excessive light energy, all it takes should be the avoidance movement. Thus, it is possible that the aggregative movement would have multiple advantages including mitigating photoinhibition and also operating efficient photosynthesis. There is a report that CO_2 fertilization does not stimulate P_n in maize in the absence of drought stress but in the presence of the stress by up to 41% (Leakey et al., 2004, 2006). Since not only light but also drought lead chloroplasts to aggregate to the BS side (Lal & Edwards, 1996; Yamada et al., 2009), the supposition that the aggregative movement of C_4-M chloroplasts contributes to the enhancement of P_n cannot be dismissed. For a better understanding of the role of the aggregative movement in the regulation of photosynthesis, comprehensive research would be needed. Findings in this study should contribute basic information to understand chloroplast behavior during the daytime and the physiological significance of the chloroplast aggregative movement in C_4 plants.

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ORCID

Eri Maai http://orcid.org/0000-0001-6178-6607

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