Journal of Experimental Botany Advance Access published February 21, 2011

Journal of Experimental Botany, Page 1 of 9
doi:10.1093/jxb/err008

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

The avoidance and aggregative movements of mesophyll chloroplasts in C_4 monocots in response to blue light and abscisic acid

Eri Maai[1], Shouu Shimada[1], Masahiro Yamada[1], Tatsuo Sugiyama[2], Hiroshi Miyake[1] and Mitsutaka Taniguchi[1,*]

[1] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi, 464-8601 Japan
[2] RIKEN Plant Science Center, Yokohama, Kanagawa, 230-0045 Japan

* To whom correspondence should be addressed: E-mail: taniguti@agr.nagoya-u.ac.jp

Received 30 October 2010; Revised 29 December 2010; Accepted 7 January 2011

Abstract

In C_4 plants, mesophyll (M) chloroplasts are randomly distributed along the cell walls, whereas bundle sheath chloroplasts are located in either a centripetal or centrifugal position. It was reported previously that only M chloroplasts aggregatively redistribute to the bundle sheath side in response to extremely strong light or environmental stresses. The aggregative movement of M chloroplasts is also induced in a light-dependent fashion upon incubation with abscisic acid (ABA). The involvement of reactive oxygen species (ROS) and red/blue light in the aggregative movement of M chloroplasts are examined here in two distinct subtypes of C_4 plants, finger millet and maize. Exogenously applied hydrogen peroxide or ROS scavengers could not change the response patterns of M chloroplast movement to light and ABA. Blue light irradiation essentially induced the rearrangement of M chloroplasts along the sides of anticlinal walls, parallel to the direction of the incident light, which is analogous to the avoidance movement of C_3 chloroplasts. In the presence of ABA, most of the M chloroplasts showed the aggregative movement in response to blue light but not red light. Together these results suggest that ROS are not involved in signal transduction for the aggregative movement, and ABA can shift the blue light-induced avoidance movement of C_4-M chloroplasts to the aggregative movement.

Key words: Abscisic acid, aggregative movement, avoidance movement, blue light, C_4 plant, chloroplast, *Eleusine coracana*, mesophyll cell, reactive oxygen species, *Zea mays*.

Introduction

C_4 plants have two types of photosynthetic cells, mesophyll (M) and bundle sheath (BS) cells, which have structurally and functionally differentiated chloroplasts. The intracellular orientation of both of the chloroplasts is also different: M chloroplasts are randomly distributed along the cell walls in most cases, whereas BS chloroplasts are located in either a centripetal (close to the vascular tissue) or centrifugal (close to the M cells) position, depending on the plant species (Dengler and Nelson, 1999). The intracellular arrangement of both types of chloroplast is acquired during cell maturation (Miyake and Yamamoto, 1987) and can be re-established 1–2 h after the disturbance of their positions by a centrifugal force (Kobayashi *et al.*, 2009). Immunofluorescence observation and pharmacological studies have revealed that C_4 plants possess a mechanism for keeping chloroplasts in the 'home' position that is dependent on the actomyosin system and cytosolic protein synthesis but not on tubulin or light (Miyake and Nakamura, 1993; Kobayashi *et al.*, 2009).

The differential positioning of C_4-M and -BS chloroplasts also occurs in response to environmental stresses. Only M chloroplasts aggregatively redistribute to the BS side in response to extremely strong light stimuli (3000–4000 μmol m^{-2} s^{-1}) or environmental stresses, such as drought, salinity, and hyperosmosis under normal light conditions (light intensity: 250–500 μmol m^{-2} s^{-1}) (Yamada *et al.*, 2009). This rearrangement of M chloroplasts also occurs in field-grown C_4 plants under midsummer drought conditions. The migration of M chloroplasts is controlled by actin filaments and is also induced in a light-dependent fashion upon incubation with abscisic acid (ABA). From these findings, it is thought that some factor generated by light irradiation functions as a trigger for the M chloroplast movement, and that ABA participates in signal transduction in response to environmental stresses. The aggregative movement of C_4-M chloroplasts is thought to be a protective response under environmental stress conditions, but the physiological significance and molecular mechanisms of the chloroplast response remain to be investigated.

In C_3 plants, the chloroplast movement in response to light intensity has been extensively studied as a photorelocation movement. Under high-intensity light irradiation, chloroplasts move away from the light to minimize photodamage, whereas under low-intensity irradiation, they move towards the light to maximize photosynthesis (Suetsugu and Wada, 2009). Blue light (BL) induces this photorelocation movement, whereas red light is also effective in some alga, moss, and fern species. Phototropin is the BL receptor for chloroplast movement in seed plants, and actin filaments are involved in the motility system (Sato and Kadota, 2007; Suetsugu and Wada, 2009). Some putative signalling and regulator proteins for the chloroplast movement have been identified from mutant analyses in *Arabidopsis thaliana*, a C_3 plant. A study with *Arabidopsis* mutants also revealed that chloroplast-avoidance movement protects plants from photodamage under strong light (Kasahara *et al.*, 2002). Under excessive light conditions, a decrease in the consumption and/or efflux of stromal reducing equivalents results in the accumulation of electrons in the photosynthetic electron transport chain, which produces harmful reactive oxygen species (ROS) that induce the photodamage (Oelze *et al.*, 2008; Foyer and Noctor, 2009; Li *et al.*, 2009; Kinoshita *et al.*, 2011). It was reported that one ROS, hydrogen peroxide (H_2O_2), is generated by high-intensity light in *Arabidopsis* M cells and promotes the chloroplast avoidance movement, and ROS are thought to be involved in the signal transduction for the avoidance movement (Wen *et al.*, 2008).

These important findings led us to investigate whether the signalling factors for the C_3-chloroplast movement also function in the intracellular movement of C_4-M chloroplasts. Compared with the light-avoidance movement of C_3-M chloroplasts, stronger light and longer exposure times are required for the aggregative movement of C_4-M chloroplasts (Yamada *et al.*, 2009). In addition, environmental stresses and ABA participate in the C_4-chloroplast movement. The intracellular directions of chloroplast movement are different between C_3 and C_4 plants, and C_4 cells might possess their own system for determining cell polarity and machinery for polarized motility. The present study was therefore aimed at validating the involvement of ROS and BL in the aggregative movement of C_4-M chloroplasts. The participation of ROS in the light-dependent M-chloroplast movement and the effects of blue and red light on the intracellular arrangement of chloroplasts in C_4 plants were investigated. From the experimental data, the relationship between the BL-induced avoidance movement and the ABA-induced aggregative movement of C_4-M chloroplasts is shown.

Materials and methods

Plant materials and growth conditions

Finger millet (*Eleusine coracana* L. Gaertn. cv. Yukijirushi), maize (*Zea mays* L. cv. Golden Cross Bantam T51), and barley (*Hordeum vulgare* cv. Haruna Nijo) were grown in vermiculite in a growth chamber with 14 h of illumination (500 μmol m^{-2} s^{-1}) at 28 °C and 10 h of darkness at 20 °C d^{-1}. Plants were fertilized regularly with Arnon and Hoagland solution (Arnon and Hoagland, 1940) during growth. The middle regions of the fully developed youngest leaf blades from 3 or 4-week-old plants were used for the experiments.

Treatment with light and chemicals

Small segments (5 mm square) were excised from the leaf blades and vacuum infiltrated for 10 min with 0.06% (v/v) ethanol with or without 30 μM ABA. Then, the leaf segments were floated on the same solution and irradiated with white light (fluorescent light, FPL27EX-N, Panasonic, Osaka, Japan), BL (LED with a maximum peak at 450 nm, ISL-150X150-BB45, CCS, Kyoto, Japan) or red light (LED with a maximum peak at 660 nm, ISL-150X150-RR, CCS). The photosynthetic photon flux density at the leaf surface was checked with a quantum meter (LI-250, Li-Cor, Lincoln, NE, USA). After incubation at 25 °C, the leaf segments were vacuum infiltrated for 15 min with fixation buffer [3% (v/v) glutaraldehyde and 50 mM Na-phosphate, pH 7.0] and incubated at 4 °C overnight. The fixed segments were inserted into a 5% (w/v) agar block and sectioned at 40–50 μm with a vibrating-blade microtome (VT1200S, Leica Microsystems GmbH, Wetzlar, Germany). Transverse sections were observed with a light microscope (BX51, Olympus, Tokyo, Japan) equipped with a CCD camera (DP70, Olympus).

For treatment with H_2O_2 and ROS scavengers, leaf segments were vacuum infiltrated with 0.06% (v/v) ethanol with or without 30 μM ABA, 0.1 mM H_2O_2, 20 mM L-ascorbate, 10 mM 1,4-diazabicyclo(2.2.2)octane (DABCO), and 5 mM 1,2-dihydroxy-3-5-benzenedisulphonate (Tiron). After floating on the same solution for 8 h under white light (100 μmol m^{-2} s^{-1}), the leaf segments were fixed and transverse sections were observed with a light microscope.

All of the experiments were repeated and the reproducibility of the obtained data was confirmed.

Quantification of the aggregative arrangement of M chloroplasts

To quantify the extent of the aggregative movement of M chloroplasts, an index of the aggregative arrangement of M chloroplasts was introduced (Fig. 1). At first, coordinates of the points A, B, C, and D in the digital image of the transverse section were measured by using a Microsoft Paint software; A (x_1, y_1), B (x_2, y_2), C (X, Y), and D (X_0, Y_0). The slope (m) and y-intercept (n) of the straight line AB are deduced from the equations (1) and (2), respectively.

$$m = \frac{y_2 - y_1}{x_2 - x_1} \qquad \text{(equation 1)}$$

$$n = y_1 - \frac{x_1(y_2 - y_1)}{x_2 - x_1} \qquad \text{(equation 2)}$$

The shortest distances between the line AB and the point C or D (d and d_0) are calculated from equations (3) and (4), respectively.

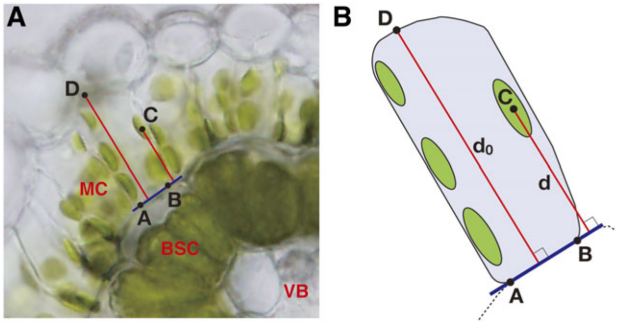

Fig. 1. Quantification of the aggregative arrangement of M chloroplasts. (A) A light microscopy image of the transverse section of finger millet leaf blade. BSC, bundle sheath cell; MC, mesophyll cell; VB, vascular bundle. (B) A diagram of an M cell in which chloroplasts are applicable to the quantitative analysis. Both ends of the M cell wall in contact with a neighbour BS cell are set as points 'A' and 'B', respectively. Within the same M cell, a point 'C' is a centre point of each chloroplast and a point 'D' is an innermost point from the straight line AB. The shortest distances between the line AB and the point C or D are set as '*d*' and 'd_0', respectively. A method for calculating the distances and index of the aggregative arrangement of M chloroplasts are described in the Materials and methods.

$$d = \frac{|Y - mX - n|}{\sqrt{1+m^2}} \qquad \text{(equation 3)}$$

$$d_0 = \frac{|Y_0 - mX_0 - n|}{\sqrt{1+m^2}} \qquad \text{(equation 4)}$$

The ratio d/d_0 is a relative distance from the BS-side cell wall to each chloroplast and the index of aggregative arrangement of M chloroplasts are deduced from equation (5).

$$\textit{index of aggregative arrangement}\left(\%\right) = \left(1 - \frac{d}{d_0}\right) \times 100 \qquad \text{(equation 5)}$$

This index indicates the extent of localization of M chloroplasts towards the BS side. The index value is calculated to be 50% when M chloroplasts are evenly distributed along the cell walls. The index value approaches 100% as M chloroplasts aggregatively migrate towards the BS side.

Results

A time-course of the ABA-induced movement of M chloroplasts

It has previously been reported that treatment of leaf segments excised from finger millet, an NAD-malic-enzyme-type C_4 plant, with ABA for 16 h induces the aggregative movement of M chloroplasts in a light-dependent fashion (Yamada *et al.*, 2009). However, a detailed time-course of the chloroplast movement was not examined. The intracellular positions of M and BS chloroplasts were observed for up to 20 h of incubation with ABA under continuous white light (100 μmol m^{-2} s^{-1}) (Fig. 2). Slight aggregative movement of M chloroplasts at the adaxial side was observed 6 h after the onset of ABA treatment, and the apparent BS-sided distribution of M chloroplasts was detected at the adaxial and abaxial sides 8 h after the beginning of the treatment. The aggregative arrangement of M chloroplasts was maintained thereafter. Leaf sections were therefore observed 8 h after the beginning of the ABA treatment in subsequent experiments. When the incubation of leaf segments was conducted in the absence of ABA or in the dark, the rearrangement of M chloroplasts was not observed until 20 h (see Supplementary Fig. S1 at *JXB* online). The centripetal arrangement of BS chloroplasts was not changed under any of the conditions.

The effect of ROS on the aggregative movement of M chloroplasts

It has been reported that exogenously applied H_2O_2 promotes the high-fluence avoidance movement of *Arabidopsis* chloroplasts in a concentration-dependent manner within the range of 0–100 μM (Wen *et al.*, 2008). To examine whether H_2O_2 stimulates the C_4-M chloroplast movement in response to ABA and light, leaf segments of finger millet were incubated with 100 μM H_2O_2 under light or dark conditions (Table 1; see Supplementary Fig. S2 at *JXB* online). When the incubation solution did not contain ABA, the aggregative movement of M chloroplasts did not occur, regardless of the presence or absence of H_2O_2. These results indicate that H_2O_2 alone does not induce the chloroplast movement. By contrast, the aggregative movement occurred regardless of the presence of H_2O_2 when the leaf segments were incubated with ABA in the light. No significant change in the extent of the chloroplast migration and the required time was observed with the H_2O_2 treatment. This ABA-induced movement was not observed under dark conditions, regardless of the presence or absence of H_2O_2. These results are evidence against the involvement of ROS in the aggregative movement of C_4-M chloroplasts. A possible relationship of ROS to the chloroplast movement

Table 1. The effects of H_2O_2 and ROS scavengers on the aggregative movement of M chloroplasts

Leaf segments excised from leaf blades of finger millet were de-aerated in 0.06% ethanol with or without 30 μM ABA, 100 μM H_2O_2, and ROS scavengers, and floated on the solution for 8 h under white light (100 μmol m^{-2} s^{-1}) or in the dark. Transverse sections were observed with a light microscope and the occurrence of the aggregative movement of M chloroplasts is listed.

Light or dark	30 μM ABA	100 μM H_2O_2	ROS scavenger	Aggregative movement
L	–	–	–	Did not occur
L	–	+	–	Did not occur
D	–	–	–	Did not occur
D	–	+	–	Did not occur
L	+	–	–	Occurred
L	+	+	–	Occurred
D	+	–	–	Did not occur
D	+	+	–	Did not occur
L	+	–	20 mM ascorbate	Occurred
L	+	–	10 mM DABCO	Occurred
L	+	–	5 mM Tiron	Occurred

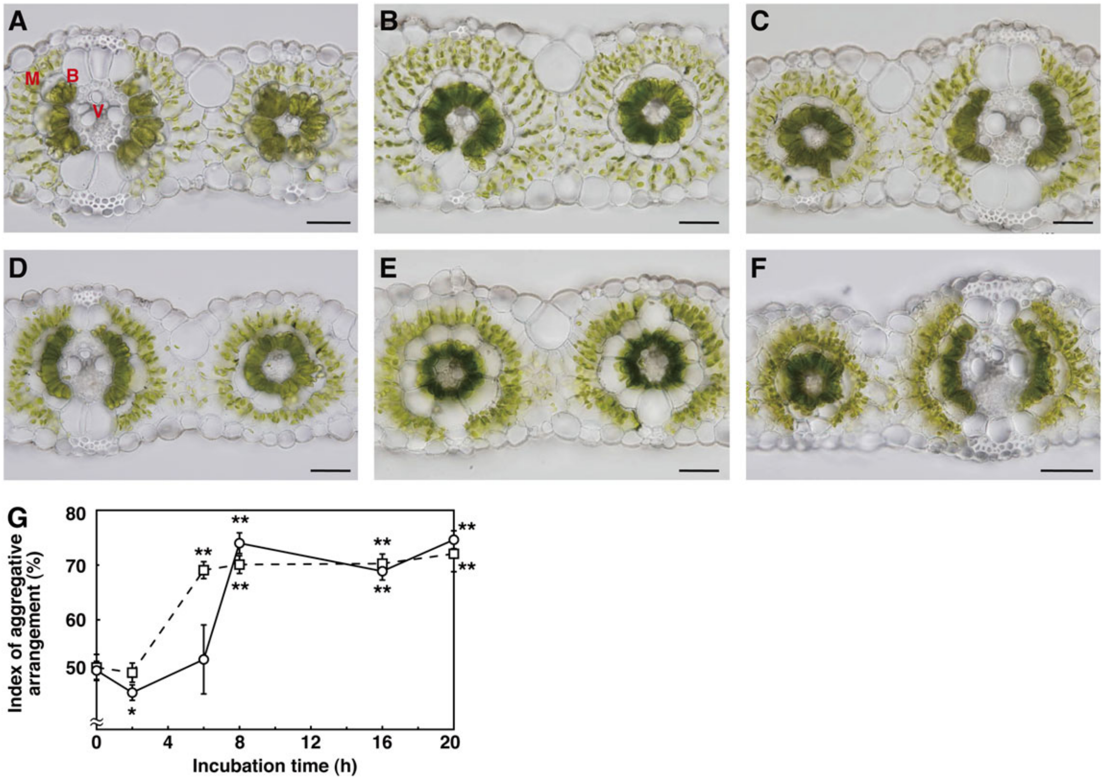

Fig. 2. The changes in the intracellular arrangement of chloroplasts in response to ABA. Leaf segments, excised from leaf blades of finger millet, were de-aerated in 0.06% ethanol with 30 μM ABA and floated on the solution under white light (100 μmol m^{-2} s^{-1}). Transverse sections were observed with a light microscope before (A) and after 2 (B), 6 (C), 8 (D), 16 (E), and 20 h (F) of illumination. The adaxial side of each leaf section (upper side in the photograph) was illuminated: B, bundle sheath cell; M, mesophyll cell; V, vascular bundle. Scale bars=50 μm. (G) The index of aggregative arrangement of M chloroplasts at the adaxial and abaxial sides (circles and squares, respectively) following illumination with ABA. Data are means ±SE (n=86–320 chloroplasts). Asterisks indicate statistically significant differences compared with t=0 according to Student's t test (*P <0.05, **P <0.01).

was examined further by using ROS scavengers. It has been reported that the application of H_2O_2 scavengers largely abolishes H_2O_2 generation and chloroplast movement in *Arabidopsis* leaves under high-intensity light (Wen *et al.*, 2008). Ascorbate was used as a scavenger of H_2O_2, DABCO as a scavenger of $^1O_2$ and Tiron as a scavenger of O_2^-, at concentrations that were reported to be effective for the suppression of ROS-induced physiological responses in rice leaves (Yamane *et al.*, 2004). If ROS participate in the C_4-M chloroplast movement, it was expected that the ABA-induced aggregative movement would be inhibited in the presence of ROS scavengers. However, it was found that the aggregative movement was not blocked by the scavenging of endogenous ROS (Table 1; see Supplementary Fig. S3 at *JXB* online). Together these findings indicate that ROS are not involved in the M chloroplast aggregative movement, in contrast to the high light-induced chloroplast-avoidance movement in *Arabidopsis*.

The effect of blue and red light on the intracellular positions of chloroplasts in finger millet

In C_3 plants, BL induces chloroplast photorelocation movements and phototropins function as BL receptors for these chloroplast movements (Suetsugu and Wada, 2009). The effect of BL on the intracellular arrangement of chloroplasts in C_4 finger millet was investigated (Fig. 3). When the leaf segments were illuminated with weak BL (20 μmol m^{-2} s^{-1}) without ABA for 8 h, an apparent rearrangement of the chloroplasts was not observed (Fig. 3A). However, in the presence of ABA, M chloroplasts aggregatively migrated towards the BS side (Fig. 3B). When BL with a light intensity of 100 μmol m^{-2} s^{-1} was applied in the absence of ABA, M chloroplasts migrated to the anticlinal walls, parallel to the direction of the incident light (Fig. 3C). This rearrangement pattern is an avoidance movement observed in C_3 leaves under high-intensity light irradiation. In the presence of ABA, the avoidance movement of M chloroplasts was observed at the illuminated side (adaxial side) of each leaf section (Fig. 3D). By contrast, the aggregative movement of M chloroplasts occurred at the opposite side (abaxial side). When high-intensity BL (500 μmol m^{-2} s^{-1}) was applied in the absence of ABA, M chloroplasts showed the avoidance movement at the illuminated side and aggregative movement at the opposite side (Fig. 3E). In the presence of ABA, the aggregative movement was observed at both the sides (Fig. 3F). These results indicate that BL can induce the avoidance movement of M chloroplasts in finger millet, and ABA promotes M chloroplasts to the

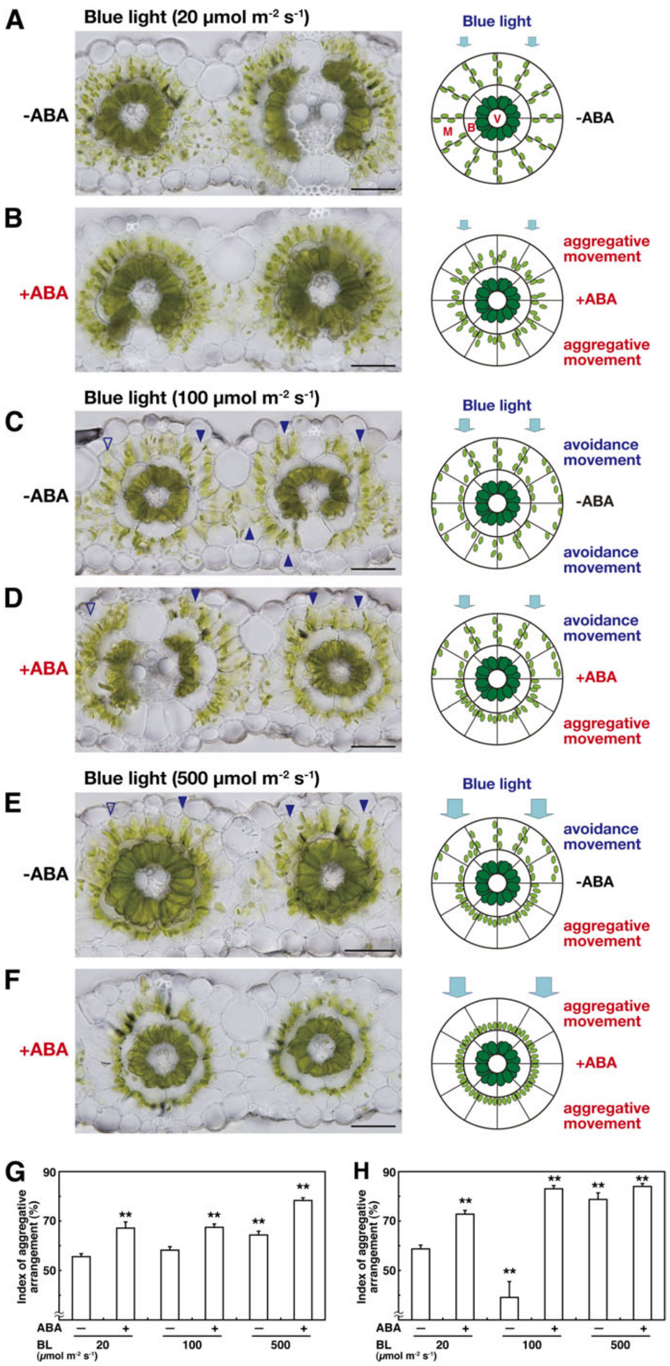

Fig. 3. The effects of blue light and ABA on the intracellular arrangement of chloroplasts. Leaf segments of finger millet were de-aerated in 0.06% ethanol with or without 30 μM ABA, floated on the solution, and continuously irradiated for 8 h with blue light (BL) of intensity 20 (A, B), 100 (C, D) or 500 μmol m^{-2} s^{-1} (E, F). The adaxial side of each leaf section (upper side in the photograph) was illuminated. Typical M cells in which chloroplasts show the avoidance response are indicated by closed arrowheads. In the same sections, there are a few M cells in which chloroplasts do not show the avoidance response and are distributed along the sides of periclinal walls, irrespective of incident direction of BL. These M cells are indicated by open arrowheads. Scale bars=50 μm. The schematic model of the chloroplast arrangement is shown on the right side of each section photograph: B, bundle sheath cell; M, mesophyll cell; V, vascular bundle. The index of aggregative arrangement of M chloroplasts at the adaxial and abaxial sides (G and H, respectively) following irradiation with BL are obtained. Data are means ±SE (n=100–355 chloroplasts). Asterisks indicate statistically significant differences compared with the treatment with BL (20 μmol m^{-2} s^{-1}) in the absence of ABA according to Student's t test (**P <0.01).

aggregative movement. These aggregative arrangements in response to BL and ABA was also confirmed by the quantitative analysis of intracellular positions of M chloroplasts (Fig. 3G, H). The index values of chloroplasts that were observed with aggregative redistribution were significantly higher than those of M cells illuminated with weak BL without ABA. It should be noted that the index value does not reflect the extent of avoidance movement of M chloroplasts. For example, the index values of M cells illuminated with BL (100 μmol m^{-2} s^{-1}) without ABA are 58% and 39% on the adaxial and abaxial sides, respectively, while the avoidance movement of M chloroplasts occurs on both sides. The centripetal arrangement of BS chloroplasts was not changed under any of the conditions.

In most C_3 plant species, red light has little effect on inducing chloroplast movement, whereas red light-induced chloroplast movement was found in some cryptogam, moss, and fern species (Suetsugu and Wada, 2009). Thus, the effect of red light on the ABA-induced aggregative movement of M chloroplasts was investigated (Fig. 4). When leaf segments of finger millet were illuminated with red light of 100 or 500 μmol m^{-2} s^{-1} intensity for 8 h, the intracellular arrangement of chloroplasts was not changed, regardless of the absence or presence of ABA.

The effect of BL and ABA on the intracellular positions of chloroplasts in maize and barley

Maize is an NADP-malic-enzyme-type C_4 plant and the BS chloroplasts are located in the centrifugal position. The M chloroplast movement was also observed in maize subjected to high-intensity white light or environmental stresses, but with a distinct pattern of redistribution along the sides of anticlinal walls, analogous to the avoidance movement of C_3 chloroplasts (Yamada *et al.*, 2009). The effect of BL and ABA on the intracellular arrangement of maize chloroplasts was examined (Fig. 5). It was observed that M chloroplasts were randomly distributed along the cell walls in leaves under normal growth conditions (white light 500 μmol m^{-2} s^{-1}) (Fig. 5A). When BL with a light intensity of 100 μmol m^{-2} s^{-1} was applied in the absence of ABA, an obvious avoidance movement of M chloroplasts occurred at both the illuminated and opposite sides (Fig. 5B). The extent of the movement was higher than that observed in maize leaves irradiated for 2 h with high-intensity white light (2000 μmol m^{-2} s^{-1}) (Yamada *et al.*, 2009). In the presence of ABA, the avoidance movement of M chloroplasts at the illuminated side and the aggregative movement at the opposite side occurred (Fig. 5C). These responses of maize

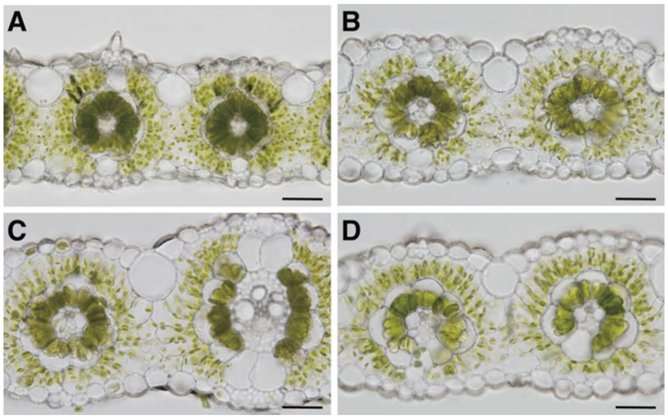

Fig. 4. The effects of red light and ABA on the intracellular arrangement of chloroplasts. Leaf segments of finger millet were de-aerated in 0.06% ethanol in the absence (A, C) or presence (B, D) of 30 μM ABA, floated on the same solutions, and continuously irradiated for 8 h with red light of intensity 100 (A, B) or 500 μmol m^{-2} s^{-1} (C, D). Scale bars=50 μm.

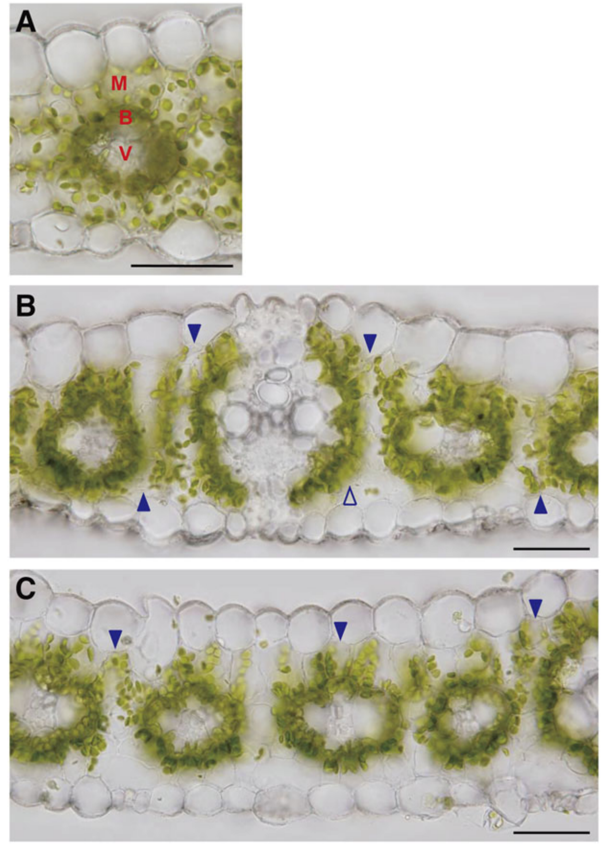

Fig. 5. The effects of blue light and ABA on the intracellular arrangement of maize chloroplasts. Leaf segments, excised from maize leaf blades, were de-aerated in 0.06% ethanol with or without 30 μM ABA and floated on the solution under blue light (100 μmol m^{-2} s^{-1}). The adaxial side of each leaf section (upper side in the photograph) was illuminated. (A) Transverse section of untreated leaf segment. (B) Transverse section of leaf segment illuminated for 8 h in the absence of ABA. (C) Transverse section of leaf segment illuminated for 8 h in the presence of ABA. Typical M cells in which chloroplasts show the avoidance response are indicated by closed arrowheads. M cells in which chloroplasts do not show the avoidance response are indicated by open arrowheads. B, bundle sheath cell; M, mesophyll cell; V, vascular bundle. Scale bars=50 μm.

M chloroplasts are similar to those of finger millet M chloroplasts (Fig. 3C, D). By contrast, the centrifugal arrangement of maize BS chloroplasts was not changed under any of the conditions.

Barley is a graminaceous C_3 monocot and multiple layers of M cells surround the single layer of BS cells. M cells contain many chloroplasts and BS cells relatively few (see Supplementary Fig. S4A at *JXB* online). M chloroplasts are randomly distributed along the cell walls and the intracellular arrangement of chloroplasts was not changed after irradiation for 8 h with BL, regardless of the absence or presence of ABA (see Supplementary Fig. S4B, C at *JXB* online). This result was quite a contrast to the response of M chloroplasts in the C_4 monocots, finger millet, and maize.

Discussion

ROS are not involved in the aggregative movement of M chloroplasts

It was reported previously that the aggregative rearrangement of C_4-M chloroplasts to the BS side is induced by environmental stresses in a light-dependent fashion, and that this movement is mediated by ABA (Yamada *et al.*, 2009). It was estimated that some factor generated by light irradiation functions as a trigger for the M-chloroplast aggregative movement, and that ABA participates in the signal transduction in response to environmental stresses. A number of studies have shown that ROS are generated under environmental stress conditions and function as signalling factors in physiological responses (Foyer and Noctor, 2009; Li *et al.*, 2009). It was hypothesized that ROS are generated in the stressed leaves by light irradiation and act as an intermediate factor in signal transduction for the light-dependent chloroplast aggregative movement in C_4-M cells. To test this possibility, the incubation experiment was conducted with H_2O_2 (Table 1). If H_2O_2 is involved in the signal transduction of the chloroplast aggregative movement in finger millet M cells, it might be possible to induce the aggregative movement by the addition of H_2O_2, even in the dark. However, the chloroplast movement did not occur under the dark conditions in the presence of H_2O_2. Moreover, exogenously applied H_2O_2 or ROS scavengers did not change the extent and required time for the ABA-induced chloroplast aggregative movement in the light. From these results, it was concluded that ROS is not a trigger or signalling intermediate for the aggregative movement of C_4-M chloroplasts. This conclusion does not agree with the previous report that H_2O_2 is involved in the high-fluence BL-induced avoidance movement of *Arabidopsis* chloroplasts (Wen *et al.*, 2008). The authors speculated that H_2O_2 generated at the plasma membrane acts on phototropin to promote the chloroplast avoidance movement. Our experimental data suggest that the signal transduction pathway for the aggregative movement of C_4-M chloroplasts is partially different from that for the avoidance movement of C_3 chloroplasts. Further studies are needed to clarify the role of ROS in chloroplast movement, making a distinction between BL-induced avoidance and the aggregative movements in C_4-M cells.

The BL-responsive avoidance movement and ABA-induced aggregative movement

Inoue and Shibata previously reported that irradiation with BL (corresponding to a light intensity of 86 μmol m^{-2} s^{-1}) induces a decrease in the absorbance of leaves from five graminaceous C_4 species, including maize, but the precise migration pattern of chloroplasts was not reported (Inoue and Shibata, 1974). In the present study, it was confirmed that BL induces the avoidance movement of M chloroplasts in finger millet and maize (Figs 3, 5). As red light was not effective in the chloroplast rearrangement (Fig. 4), it is concluded that the avoidance and aggregative movements of C_4-M chloroplasts are mediated by a BL receptor, such as phototropin. The response of M chloroplasts to BL in the absence of ABA varied by the intensity of BL (Fig. 3A, C, E). The avoidance movement was not observed at a weak BL intensity (20 μmol m^{-2} s^{-1}) but was observed at a BL intensity above 100 μmol m^{-2} s^{-1}. The avoidance movement was observed at the illuminated and opposite sides in leaves that were exposed to BL, with the light intensity of 100 μmol m^{-2} s^{-1}, whereas high-intensity BL (500 μmol m^{-2} s^{-1}) induced the avoidance movement at the illuminated side and the aggregative movement at the opposite side. Because ABA can induce the chloroplast aggregative movement, there is speculation that the illumination by high-intensity BL may promote the synthesis or relocalization of ABA at the unirradiated side and promote the ABA-induced aggregative movement of M chloroplasts.

In the presence of ABA, the aggregative movement of M chloroplasts was observed on both sides of the leaves irradiated with the three kinds of light intensity (Fig. 3B, D, F). The only exception was the avoidance movement observed at the illuminated side of leaves exposed to BL with the light intensity of 100 μmol m^{-2} s^{-1} (Fig. 3D). At present, the reason cannot be explained, but it is possible that the balance between the BL intensity and ABA concentration may be important for the aggregative movement to occur. The distribution of M chloroplasts encircling BS cells in response to ABA occurred even under the weak BL (20 μmol m^{-2} s^{-1}), whereas white light with an equal light intensity could not induce the chloroplast movement (data not shown). The distribution of the chloroplasts encircling the BS cells became more compact with an increase in BL intensity (Fig. 3G, H). These results suggest that ABA (or an unknown ABA-derived factor) can shift M chloroplasts from the BL-induced avoidance distribution to the aggregative distribution. In field-grown C_4 plants, high-intensity sunlight, including BL, may essentially elicit the avoidance movement of M chloroplasts to provide photoprotection through the mutual shading of the chloroplasts, similar to C_3 chloroplasts. If the C_4 plants receive severe environmental stresses such as drought or salinity stress, ABA may be synthesized in the leaves, or synthesized in roots and transported to leaves, and then ABA may shift M chloroplasts to the aggregative movement for the protection of BS chloroplasts against photodamage and/or the maintenance of C_4 photosynthetic activity. Encircling BS cells by M chloroplasts shortens the diffusion pathway of metabolites between M and BS cells, and may contribute to keeping photosynthesis active. Alternatively, it may be advantageous to refix the CO_2 released from BS cells (Yamada *et al.*, 2009). It has been reported that maize M chloroplasts collapse inward and aggregate in synchrony with inhibition of photosynthesis under the water stress conditions (Lal and Edwards, 1996). The aggregative arrangement of M chloroplasts naturally occurs in field-grown C_4 plants when exposed to high-intensity sunlight and drought stress (Yamada *et al.*, 2009). Chloroplast rearrangement in response to ABA has not been found in a C_3 monocot, barley and, therefore, the aggregative movement of chloroplasts might be an adaptive strategy of C_4 plants to survive under dry and high-temperature conditions. In our experiment, BS chloroplasts showed no response to BL and ABA. It is thought that C_4 BS cells possess a mechanism for anchoring chloroplasts in the centripetal or centrifugal position (Kobayashi *et al.*, 2009). However, there is a report that maize BS chloroplasts lose their centrifugal position and become equally distributed around the cell in response to water stress (Lal and Edwards, 1996). The relationship between the intracellular positioning of BS chloroplasts and environmental stress remains to be investigated.

It was previously reported that maize M chloroplasts redistribute along the sides of anticlinal walls, analogous to the C_3 avoidance movement, in response to high-intensity white light (Yamada *et al.*, 2009). In this study, BL (100 μmol m^{-2} s^{-1}) induced an obvious light avoidance movement of maize M chloroplasts (Fig. 5). The exogenously applied ABA shifted the aggregative arrangement at the opposite side of BL irradiation, similar to the observation in finger millet. The aggregative rearrangement of M chloroplasts in response to drought or salinity stresses was confirmed in some phospho*enol*pyruvate carboxykinase-type C_4 species (S Shimada *et al.*, unpublished data). Therefore, it is thought that the mechanism of M-chloroplast movement is conserved among the three subtypes of C_4 plants.

To summarize the properties of the chloroplast photorelocation movement in C_4 and C_3 plants (Table 2), extremely high-intensity white light induces chloroplast aggregative movement in C_4 plants. This response is specific to M chloroplasts, and strong light and long exposure times are required to elicit a response, as compared with the light-avoidance movement of M chloroplasts in C_3 plants (Yamada *et al.*, 2009). In addition, the patterns of the chloroplast rearrangement in C_4-M cells in response to

Table 2. Comparison of the properties of chloroplast relocation movement in C_4 and C_3 plants

	C_4 plants[a]	C_3 plants[a]
Response to high-intensity white light	M chloroplast-specific aggregative movement or avoidance movement (depending on the plant species) (1)	Avoidance movement
Response to blue light	Yes	Yes
Response to red light	No	No/yes (moss, fern) (2)
Response to environmental stress	Yes (1) Drought (3,4), Salinity, Hyperosmosis (light-dependent manner)	Yes Mechanical stress (5), Low temperature (maidenhair fern) (6), Submergence (marsh dock) (7), Bacterial elicitor (tobacco) (8)
Involvement of ROS	No	H_2O_2 (promoting effect) (9)
Involvement of plant hormone	ABA (light dependent) (1)	No report
Involvement of actin filament	Yes (1)	Yes
Interaction with mitochondria and nuclei	Independent (1)	Co-localize? (10, 11, 12)

[a] (1) Yamada *et al.*, 2009, (2) Suetsugu and Wada, 2009, (3) Lal and Edwards, 1996, (4) Guralnick *et al.*, 2002 (5) Sato *et al.*, 1999, (6) Kodama *et al.*, 2008, (7) Mommer *et al.*, 2005, (8) Boccara *et al.*, 2007, (9) Wen *et al.*, 2008, (10) Iwabuchi *et al.*, 2007, (11) Tsuboi *et al.*, 2007, (12) Islam *et al.*, 2009.

environmental stresses are dependent on the plant species, as the avoidance movement of M chloroplasts in response to high-intensity light preferentially occurs in maize. The avoidance movement of M chloroplasts also occurs in finger millet leaves exposed to BL, but ABA shifts the chloroplast disposition to the aggregative manner. These responses to BL and ABA are unique to C_4-M chloroplasts, in addition to the responses to environmental stresses and no role of ROS. During the evolution of C_4 plants, M cells have acquired a mechanism for the aggregative movement of chloroplasts in addition to the avoidance movement and BS cells have a mechanism for holding chloroplasts in the centripetal or centrifugal position. Further research should explore the mechanisms for C_4 chloroplast arrangement and an attempt to provide the mechanisms to C_3 plants for improving their ability of stress tolerance.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. The effect of white light and ABA on the intracellular arrangement of chloroplasts.

Supplementary Fig. S2. The effect of H_2O_2 on the intracellular arrangement of chloroplasts.

Supplementary Fig. S3. The effect of ROS scavengers on the intracellular arrangement of chloroplasts.

Supplementary Fig. S4. The effects of blue light and ABA on the intracellular arrangement of barley chloroplasts.

Acknowledgement

This work was supported by the Japan Society of the Promotion of Science (No. 21380014 to MT).

References

Arnon DI, Hoagland DR. 1940. Crop production in artificial solutions and soils with special reference to factors influencing yield and absorption of inorganic nutrients. *Soil Science* **50,** 463–471.

Boccara M, Schwartz W, Guiot E, Vidal G, De Paepe R, Dubois A, Boccara AC. 2007. Early chloroplastic alterations analysed by optical coherence tomography during a harpin-induced hypersensitive response. *The Plant Journal* **50,** 338–346.

Dengler NG, Nelson T. 1999. Leaf structure and development in C_4 plants. In: Sage RF, Monson RK, eds. *C_4 plant biology*. San Diego: Academic Press, 133–172.

Foyer CH, Noctor G. 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxidants nd Redox Signaling* **11,** 861–905.

Guralnick LJ, Edwards G, Ku MSB, Hockema B, Franceschi VR. 2002. Photosynthetic and anatomical characteristics in the C_4-crassulacean acid metabolism-cycling plant, *Portulaca grandiflora*. *Functional Plant Biology* **29,** 763–773.

Inoue Y, Shibata K. 1974. Comparative examination of terrestrial plant leaves in terms of light-induced absorption changes due to chloroplast rearrangements. *Plant and Cell Physiology* **15,** 717–721.

Islam MS, Niwa Y, Takagi S. 2009. Light-dependent intracellular positioning of mitochondria in *Arabidopsis thaliana* mesophyll cells. *Plant and Cell Physiology* **50,** 1032–1040.

Iwabuchi K, Sakai T, Takagi S. 2007. Blue light-dependent nuclear positioning in *Arabidopsis thaliana* leaf cells. *Plant and Cell Physiology* **48,** 1291–1298.

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M. 2002. Chloroplast avoidance movement reduces photodamage in plants. *Nature* **420,** 829–832.

Kinoshita H, Nagasaki J, Yoshikawa N, Yamamoto A, Takito S, Kawasaki M, Sugiyama T, Miyake H, Weber APM, Taniguchi M. 2011. The chloroplastic 2-oxoglutarate/malate transporter has dual function as the malate valve and in carbon/nitrogen metabolism. *The Plant Journal* **65,** 15–26.

Kobayashi H, Yamada M, Taniguchi M, Kawasaki M, Sugiyama T, Miyake H. 2009. Differential positioning of C_4 mesophyll and bundle sheath chloroplasts: recovery of chloroplast positioning requires the actomyosin system. *Plant and Cell Physiology* **50,** 129–140.

Kodama Y, Tsuboi H, Kagawa T, Wada M. 2008. Low temperature-induced chloroplast relocation mediated by a blue light receptor, phototropin 2, in fern gametophytes. *Journal of Plant Research* **121,** 441–448.

Lal A, Edwards GE. 1996. Analysis of inhibition of photosynthesis under water stress in the C_4 species *Amaranthus cruentus* and *Zea mays*: electron transport, CO_2 fixation and carboxylation capacity. *Australian Journal of Plant Physiology* **23,** 403–412.

Li ZR, Wakao S, Fischer BB, Niyogi KK. 2009. Sensing and responding to excess light. *Annual Review of Plant Biology* **60,** 239–260.

Miyake H, Nakamura M. 1993. Some factors concerning the centripetal disposition of bundle sheath chloroplasts during the leaf development of *Eleusine coracana*. *Annals of Botany* **72,** 205–211.

Miyake H, Yamamoto Y. 1987. Centripetal disposition of bundle sheath chloroplasts during the leaf development of *Eleusine coracana*. *Annals of Botany* **60,** 641–647.

Mommer L, Pons TL, Wolters-Arts M, Venema JH, Visser EJW. 2005. Submergence-induced morphological, anatomical, and biochemical responses in a terrestrial species affect gas diffusion resistance and photosynthetic performance. *Plant Physiology* **139,** 497–508.

Oelze ML, Kandibinder A, Dietz KJ. 2008. Redox regulation and overreduction control in the photosynthesizing cell: complexity in redox regulatory networks. *Biochimica et Biophysica Acta* **1780,** 1261–1270.

Sato Y, Kadota A. 2007. Chloroplast movement in response to environmental signals. In: Robert RW, Hoober K, eds. *The structure and function of plastids*. Heidelberg: Springer, 527–537.

Sato Y, Kadota A, Wada M. 1999. Mechanically induced avoidance response of chloroplasts in fern protonemal cells. *Plant Physiology* **121,** 37–44.

Suetsugu N, Wada M. 2009. Chloroplast photorelocation movement. In: Sandelius AS, Aronsson H, eds. *The chloroplast: interactions with the environment*, Vol. 13. Berlin, Heidelberg: Springer-Verlag, 235–266.

Tsuboi H, Suetsugu N, Kawai-Toyooka H, Wada M. 2007. Phototropins and neochrome1 mediate nuclear movement in the fern *Adiantum capillus-veneris*. *Plant and Cell Physiology* **48,** 892–896.

Wen F, Xing D, Zhang LR. 2008. Hydrogen peroxide is involved in high blue light-induced chloroplast avoidance movements in *Arabidopsis*. *Journal of Experimental Botany* **59,** 2891–2901.

Yamada M, Kawasaki M, Sugiyama T, Miyake H, Taniguchi M. 2009. Differential positioning of C_4 mesophyll and bundle sheath chloroplasts: aggregative movement of C_4 mesophyll chloroplasts in response to environmental stresses. *Plant and Cell Physiology* **50,** 1736–1749.

Yamane K, Rahman S, Kawasaki M, Taniguchi M, Miyake H. 2004. Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.). *Plant Production Science* **7,** 292–300.