Bundle sheath cell-dependent chloroplast movement in mesophyll cells of C_4 plants analyzed using live leaf-section imaging

Yuta Kato[1], Takao Oi[1] Yoshikatsu Sato*[2] and Mitsutaka Taniguchi*[1]

[1] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.*

[2] *Institute of Transformative Bio-Molecules, Nagoya University, Nagoya, 464-8601 Japan*

Current affiliation of Yuta Kato: *Graduate School of agricultural Sciences, Kyoto University, Kyoto, 606-8502, Japan.*

Email addresses of each author:

Yuta Kato: *kato.yuta.3e@kyoto-u.ac.jp*

Takao Oi: *oitaka@agr.nagoya-u.ac.jp*

Yoshikatsu Sato: *sato.yoshikatsu.h7@f.mail.nagoya-u.ac.jp* * *Co-Correspondence*

Mitsutaka Taniguchi: *taniguti@agr.nagoya-u.ac.jp* * *Correspondence*

Date of submission:

Number of figures: 5

Number of tables: 2

Word count: 2717

Number of supplementary figures: 6

Number of supplementary tables: 0

Number of supplementary movies: 7

Running title: Intercellular response of chloroplast movement in C_4 plant.

Highlight

We established live leaf-section imaging to observe individual chloroplast movements in multi-layered cells and found that bundle sheath cells are involved in the aggregative movement of mesophyll chloroplasts.

Abstract

In C_4 plants, mesophyll (M) chloroplasts aggregate toward bundle sheath (BS) cells in response to environmental stress, which would contribute to C_4 photosynthetic cycle adjustment between M and BS cells. However, it remains unclear whether mesophyll chloroplast movement is an intercellular response mediated by BS cells. One major challenge to resolving this is the difficulty in observing how M chloroplasts aggregate toward adjacent BS cells due to scattering and absorption of observation light in live-leaf tissues. We established a live leaf-section imaging technique that enables the long-term observation of sections of chemically unfixed leaf blades, with which we quantitatively analyzed M chloroplast movements. Another challenge in clarifying the contribution of BS cells to M chloroplast movement is the selective ablation of BS cells without impairing their function of M cells. To investigate the necessity of BS cells for M chloroplast movement, we developed a method to remove BS cells only based on differences in shape and size between M and BS cells. We also found that chloroplasts in M cells without adjacent BS cell contents did not show typical aggregative movement but showed a light-avoidance response. This indicates that the M chloroplast aggregative movement occurs during communication with BS cells.

Keywords: Aggregative movement, bundle sheath cell, C_4 photosynthesis, *Eleusine coracana*, mesophyll cell, chloroplast movement, live imaging.

Abbreviations: bundle sheath (BS), mesophyll (M), relative distance (*RD*).

53 **Introduction**

54 C_4 plants have leaves composed of multiple cell layers, including two types of photosynthetic
55 cells, mesophyll (M) and bundle sheath (BS) cells. These two cell types form a concentric
56 arrangement called "Kranz anatomy" (Brown, 1975). The C_4 photosynthetic cycle is driven across
57 both cell types and serves as a pump to concentrate and supply CO_2 to Rubisco within the BS
58 chloroplasts (Hatch, 1999), thereby improving photosynthetic efficiency by suppressing
59 photorespiration (Sage *et al*., 2012). In C_4 leaves, M chloroplasts are fewer than in C_3 leaves and
60 lack Rubisco, whereas BS chloroplasts are larger than M chloroplasts and contain Rubisco (Stata
61 *et al*., 2014). Regarding the intracellular arrangement of chloroplasts, chloroplasts in the BS cells
62 are located in either a centripetal (close to the vascular tissue) or centrifugal (close to the M cells)
63 position, depending on the plant species (Dengler and Nelson, 1999). In contrast, M chloroplasts
64 are discretely distributed along the plasma membrane throughout the cell under no-stress
65 conditions. In contrast, they aggregate toward the BS side of M cells in response to environmental
66 stresses, such as drought, salinity, hyperosmosis, and intense light, whereas BS chloroplasts
67 hardly move under these stress conditions (Lal and Edward, 1996; Yamada *et al*., 2009; Maai *et*
68 *al*., 2011b; Taniguchi and Cousins, 2018; Maai *et al*., 2020; Kato *et al*., 2022; 2023). The
69 directional movement of C_4 M chloroplasts is known as "aggregative movement". This
70 chloroplast movement depends on actin filaments and induced by blue light but not red light
71 (Kobayashi *et al.*, 2009; Yamada *et al*., 2009; Maai *et al*., 2011b). Chloroplast aggregative
72 movement is thought to contribute to the efficiency of intercellular metabolite transport and the
73 remobilization of leaked CO_2 by shortening the distance between M chloroplasts and BS cells
74 under stress conditions (Yamada *et al.*, 2009; Maai *et al.*, 2011a; 2020; Kato *et al.*, 2023).
75 However, there is no evidence suggesting that the movement of chloroplasts in M cells is due to
76 intercellular communication with BS cells.

77 Methods for studying chloroplast movement in live plants are well established,
78 particularly for analyzing chloroplast photo-relocation movements in response to light intensity

79 and wavelength (Senn, 1908; Wada *et al*., 2003; Wada, 2013; Wada and Kong, 2018).
80 Transmission measurements and band assays (Inoue and Shibata, 1973; Kagawa *et al*., 2001;
81 Wada, 2013), polarized light irradiation (Haupt and Scheuerlein, 1990), and microbeam
82 irradiation (Wada *et al.*, 1983; Wada, 2013) have been used to elucidate the mechanisms and
83 significance of chloroplast movement. However, these techniques are challenging to apply to
84 chloroplast aggregative movements, which include moving in the vertical direction relative to the
85 leaf surface within the multilayered, complex structures of C_4 leaves. Therefore, previous studies
86 on chloroplast aggregative movements in C_4 plants have relied on observing leaf cross-sections
87 prepared after chemical fixation (Yamada *et al*., 2009; Kato *et al*., 2023). This method has not
88 accurately captured chloroplast movement in the living state. There has long been a need to
89 develop a technique to observe how M chloroplasts move toward BS cells within complex leaf
90 tissue to elucidate the mechanism of chloroplast aggregative movement.

91 In this study, we developed a useful live leaf-section imaging method that analyzes
92 individual behaviors of M chloroplasts in C_4 plants. Using this method, we quantitatively analyzed
93 M chloroplasts based on their distance from adjacent BS cells. Furthermore, we succeeded in
94 preparing sections that excluded BS cells using a precise sectioning technique and investigated
95 M chloroplast behavior in the absence of BS cells. Based on these investigations, we found that
96 the aggregative movement of M chloroplasts requires intercellular communication with BS cells.
97

98 **Materials and Methods**

99 *Plant material and growth conditions*

100 Seeds of finger millet (*Eleusine coracana* L. Gaertn.) was purchased from Snow Brand Seed Co.
101 Ltd., Japan, sterilized in 5% (w/v) sodium hypochlorite solution for 5 min, and imbibed on wet
102 filter paper. Seeds were germinated in a growth chamber under a 14 h photoperiod (08:00–22:00)
103 with a temperature of 28/20 °C (day/night). The PPFD was approximately 400 μmol m^{-2} s^{-1} with
104 metal halide lamps (MF400GC/BUD, Koito Co. Ltd., Japan). The relative humidity was

105 maintained at more than 60% all day. Germinated seedlings were then transplanted into 100 mL
106 pots filled with culture soil (Hanachan Baiyodo Premium; Hanagokoro, Japan) based on bark
107 compost mixed with coconut fiber, humic minerals, and Kanuma soil and grown in the same
108 chamber for approximately two weeks. The uppermost fully expanded leaves (seventh leaf) were
109 used in the experiments, and all treatments were started between 09:30 and 10:30.
110
111 *Live leaf-section imaging*
112 The 5 mm square segments were excised from the leaf blades and vacuum-infiltrated for 5 min
113 with purified water. The leaf segments were embedded in 5% (w/v) melted agar. The agar was
114 chilled and stirred during the embedding process until immediately before solidification to
115 prevent heat damage to the leaf segments. The leaf segment embedded in the agar was cut into a
116 100 μm thick transverse section using a vibrating blade microtome (VT1200S; Leica
117 Microsystems, Germany). The obtained leaf section was carefully moved in a water droplet made
118 with the surface tension of water at the tips of tweezers and placed in a narrow groove filled with
119 0.1% agar solution in clear silicone rubber on a glass slide (Supplementary Figure S1A). The
120 adaxial or abaxial side of the leaf section was made to face the incident direction of blue light
121 (Supplementary Figure S1B). Agar was added to the mounting solution to reduce fluidity and
122 prevent the shifting of leaf sections during observation. In addition to the narrow groove in which
123 the leaf section was placed, a large square groove was incorporated into the silicone rubber
124 (Supplementary Figure S1A). This large square groove reduces the flow of the solution and
125 prevents the leaf section from flowing out of the groove when a cover glass is used. The mounted
126 leaf section was observed at 25°C under red observation light (< 1 μmol m^{-2} s^{-1}) and snapped per
127 1 min with a microscope (BX51, Olympus, Japan) equipped with a CMOS camera (DP74;
128 Olympus, Japan) (Supplementary Figure S1C). Red light filters that cut light below 600 nm were
129 placed after the halogen lamp and in front of the camera in the optical path of the microscope
130 light. Blue LED light (500 μmol m^{-2} s^{-1}, maximum peak at 450 nm; ISL-150X150-BB45, CCS

131 Inc., Japan) irradiated to the side of the mounted sample. As the position of the leaf section easily
132 changed immediately after setting the cover glass, microscopic pre-observations were conducted
133 for at least 1 h to ensure that the shift of the leaf section stabilized before starting the experiments.
134
135 *Removal of contents of BS cells and observation of chloroplast movement*
136 Small segments of leaf blade embedded in agar were cut into longitudinal sections with intact M
137 cells at 40 μm thickness to remove the BS cell contents or 60 μm thickness to keep the BS cell
138 contents using the vibrating blade microtome. From these longitudinal sections, those containing
139 vascular tissue were selected for subsequent live imaging, as described above.
140
141 *Quantification of M chloroplast arrangement*
142 We calculated the relative distance (*RD*) of the M chloroplasts from the BS side to evaluate the
143 degree of chloroplast aggregation at each time point, as previously described (Kato *et al*., 2023;
144 Supplementary Figure S4A). In this study, we used the *RD* value as an index of the position of
145 each chloroplast in an M cell, as calculated from the digital images of the sections. The pixel
146 coordinates of the points were measured using Katikati Counter software (GTSOFT, Japan).
147
148 *Quantification of M chloroplast moving*
149 To perform a tracking analysis of the movement of each chloroplast, we utilized the "Manual
150 Tracking" plugin available in Fiji (Schindelin *et al*., 2012). Tracking intervals were every minute,
151 and tracking was aborted when each chloroplast was no longer recognizable because of overlap
152 with other chloroplasts or was out-of-focus because of chloroplast movement. In addition, we
153 obtained "*Total movement*" to quantify the extent of chloroplast movement in any direction and
154 "*Displacement*" to quantify the extent of chloroplast movement toward the BS cells during 1 h
155 after blue light irradiation (Fig. 3A). In addition, *Displacement/Total movement* was calculated to
156 represent the proximity to the BS cell side. Correlations were determined between these

157 parameters and chloroplast position at the initial of blue light irradiation "*Initial position*" to
158 determine if the distance from BS cells affects the movement of M chloroplasts. The numbers of
159 measured chloroplasts and other information are presented in the figure legends.
160
161 *Statistical analyses*
162 Data were analyzed using Microsoft Excel with the add-in software Statcel 3 (OMS Publishing,
163 Japan). The significance of differences among the medians of *RD* values for each treatment was
164 estimated using Student's *t*-test or the Tukey–Kramer method. The importance of differences
165 among M chloroplast motility for each treatment was estimated using the Mann–Whitney *U* or
166 Steel–Dwass tests. The correlation between M chloroplast motility and chloroplast position at the
167 initial treatment stage was estimated using Pearson's correlation coefficient. The details of the
168 statistical parameters are provided in the figure legends.
169
170
171 **Results**
172 ***Establishment of live leaf-section imaging to continuously observe chloroplast movement in***
173 ***the C_4 plants, Eleusine coracana* L. Gaertn.**
174 To observe chloroplast movement within mesophyll tissues in C_4 plants, a transverse section of
175 the *E. coracana* leaf blade, without chemical fixation, was set in a narrow groove in a transparent
176 silicone rubber on a glass slide and irradiated with blue light from the adaxial side to induce
177 chloroplast movement (Supplementary Figure S1). This process has three critical points: handling
178 of sliced leaves, immobilization of leaves during preparation, and the light irradiation method.
179 First, preserving the leaf morphology and avoiding damage from drying or contact was crucial
180 when leaf sections were moved from the microtome stage to their proper position in the grooves
181 on glass slides. Therefore, the leaf sections were transferred using water droplets formed by the
182 surface tension of water at the tips of tweezers. It is also essential to prevent sample drift during

183 prolonged observations. To address this issue, we modified the grooves' shape and increased the
184 mounting solution's viscosity by adding agar (Supplementary Figure S1A, B). Transverse sections
185 of the living leaf tissue maintained a typical Kranz anatomy; M cells surrounded the BS cells with
186 a ring-like layer (Fig. 1). M chloroplasts were dispersed throughout the cell, whereas the BS
187 chloroplasts occupied centripetal positions on the vein side of the BS cell without light stimulation
188 (Fig. 1A). Finally, the responses to the observation and stimulus lights should be distinguished.
189 Therefore, we used red light, which does not induce chloroplast movement, for observation,
190 whereas blue light was used to induce chloroplast movement from the epidermal side of the leaves
191 using an external light source (Supplementary Figure S1C). Time-lapse observations
192 demonstrated that the M chloroplasts aggregated toward adjacent BS cells upon blue light
193 irradiation (Supplementary Movie S1), whereas they did not show this aggregative movement in
194 the absence of blue light (Supplementary Figure S2A; Supplementary Movie S2). Quantitative
195 analysis of the time-lapse images showed that the aggregative response reached a maximum at
196 approximately 4 h and was maintained even after 16 h (Supplementary Figures S3A, S4). In
197 contrast, BS chloroplasts maintained their positions centripetal to the vein throughout the
198 experimental period. When the blue irradiation was stopped after 4 h, the aggregated chloroplasts
199 gradually dispersed to a state similar to that before blue irradiation (Supplementary Figure S5A;
200 Supplementary Movie S3). These results indicated that our leaf-section imaging method
201 unequivocally minimized the effects of slicing injury and enabled, for the first time, sufficiently
202 long observations of chloroplast movement in C_4 plants.
203
204 ***Similar responsiveness of chloroplast movement in the adaxial and abaxial leaf tissues***
205 Focusing on the light incident direction on the leaf, M chloroplasts on the adaxial side of the leaf
206 tissues moved remarkably closer to the BS cells under blue light irradiation from the adaxial side
207 (Supplementary Movie S1). In contrast, those on the abaxial side moved remarkably closer to the
208 BS cells when blue light irradiation was applied from the abaxial side (Supplementary Movie S4).

These results indicate no significant difference in the reactivity of M cells between the adaxial and abaxial sides of the leaf. Instead, the direction of light incidence strongly influences them, and M cells on the side of light incidence show high responsiveness.

Blue light accelerates the speed of M chloroplast movement toward BS cells

To quantitatively evaluate how M chloroplasts move toward the BS cells, we tracked individual M chloroplasts from time-lapse images acquired every minute (Fig. 2; Supplementary Movie S5; Supplementary Figures S2B, C; S3B; S5B, C). We quantitatively evaluated the chloroplast movements in each experiment for 1 h when the chloroplasts moved actively. In the blue light irradiation experiment (Supplementary Movie S1), 1 h after the initial irradiation; in the no blue light irradiation experiment (Supplementary Movie S2), 1 h after the initial observation; and in the turn-off of blue light irradiation experiment (Supplementary Movie S3), 1 h after the irradiation was turned off. We found that the speed of M chloroplast movement toward BS cells was significantly faster than that in the absence of blue light irradiation and turn-off of blue light irradiation (Table 1). Although this trend was more pronounced on the adaxial side, it was similar on both sides. Therefore, subsequent experiments and analyses were conducted only on the adaxial sides of the leaf sections.

In addition, we investigated whether the aggregation of M chloroplasts was influenced by their initial distance from adjacent BS cells. The correlation between chloroplast behavior and distance from the BS cells at the initial irradiation (*Initial position*) was analyzed. It was found that there were weak positive correlations between *Initial position* and *Total movement* or *Displacement*, but not *Displacement/Total movement* (Fig. 3). These trends indicate that M chloroplasts that were located farther from the BS cells and near the light source appeared to move more toward the BS cells. The distance from the BS cells did not seem to affect the rate of approaching toward the BS cells in terms of total migration.

235

236 ***Necessity of BS cells for M chloroplast aggregative movements***

237 We investigated whether BS cells were necessary for the movement of M chloroplasts toward BS
238 cells by deactivating BS cell function. First, we attempted to selectively disrupt BS cells using a
239 laser microbeam dissection system (Supplementary Figure S6). Because M and BS cells were
240 adjacent, however, adjusting the light intensity to burst only BS cells was impossible. Therefore,
241 as an alternative method for examining the behavior of M chloroplasts in the absence of BS cells,
242 we focused on the differences in cell shape and attempted to remove only the BS cells. A ring
243 structure of M and BS cells around the vein was observed in transverse sections (Figs. 4A, B). In
244 contrast, M cells, BS cells, and vascular bundles were observed as stacked layers in longitudinal
245 sections (Figs. 4A, C). The width of BS cells was larger than that of M cells in the transverse
246 section (Fig. 4B). Therefore, a longitudinal section without BS cell contents was obtained by
247 longitudinally cutting thinner than the BS cell and thicker than the M cell. Sections with BS cell
248 contents were obtained by adjusting the thickness to 60 μm, while those without BS cell contents
249 were obtained by setting the thickness to 40 μm. (Figs. 4D, E). In the M cells adjacent to the intact
250 BS cells that maintained their cellular contents, the chloroplasts moved toward the BS cells and
251 densely congregated at the junction between the M and BS cells under blue light irradiation from
252 the adaxial side (Fig. 5A; Supplementary Movie S6). However, chloroplasts did not show typical
253 aggregative movement in M cells adjacent to the dead BS cells without their cellular contents.
254 Instead, they were aligned parallel to the direction of blue light incidence, moving away from the
255 incident direction, which was reminiscent of the light avoidance response (Figs. 5B;
256 Supplementary Movie S7). In sections with and without BS cell contents, when blue light
257 irradiation was stopped, the M chloroplasts gradually dispersed to a state similar to that before
258 blue irradiation. The finding that chloroplasts in M cells showed aggregative movement in
259 response to blue light only when intact BS cells were adjacent to the M cells was also statistically
260 confirmed (Fig. 5C). Although there was no significant difference in the speed of M chloroplast

261 movement between with and without BS cell contents, M chloroplasts with BS cell contents
262 significantly more oriented to the BS cells than without BS cell contents (Table 2). These results
263 indicate that BS cells are necessary for the movement of M chloroplasts toward BS cells in
264 response to blue light.
265
266 **Discussion**
267 ***Live leaf-section imaging allows continuous observation of chloroplast movement in the C_4***
268 ***leaf***
269 Previous studies on chloroplast aggregative movement in C_4 plants have compared chloroplast
270 arrangements in leaf sections after chemical fixation under different conditions (Yamada *et al*.,
271 2009, Maai *et al*., 2011b; Kato *et al*., 2023). In this study, we successfully established, for the first
272 time, a live leaf-section imaging method that enables continual observation of chloroplast
273 aggregative movements in M cells located inside the leaf tissues of the C_4 plant finger millet (Fig.
274 1). The degree of M chloroplast arrangement toward the BS cells in response to intense blue light
275 irradiation reached a maximum at approximately 4 h and was maintained until at least 16 h later
276 (Supplementary Figures S3A; S4; Supplementary Movie S1). In previous experiments in which
277 leaf fragments and fresh leaves were exposed to blue light, pronounced chloroplast aggregative
278 movement occurred within 4 to 8 h, comparable to the movement observed in live leaf sections
279 in the present study (Maai *et al*., 2011b; Kato *et al*., 2023). Furthermore, M chloroplasts returned
280 to a dispersed arrangement after stopping blue light irradiation (Supplementary Figure S5;
281 Supplementary Movie S3), indicating that the reversible responsiveness of chloroplast movement
282 was retained in the leaf sections. Although our method requires careful consideration of the fact
283 that samples were exposed to conditions different from the typical growth environment, such as
284 loss of connectivity with surrounding cells and placement in a liquid-phase environment, it can
285 be considered that the physiological response mechanisms related to chloroplast movement were
286 maintained. Therefore, our leaf-section imaging technique, which allows for observing

287 chloroplast movement inside the leaf tissue of C_4 plants, is considered an effective method for
288 analyzing chloroplast movement.
289
290 ***M chloroplast aggregative movement occurs through communication with BS cells***
291 M chloroplast aggregative movement is considered a physiological response associated with C_4
292 photosynthesis, which functions between M and BS cells because the directionality of movement
293 is toward the BS cells (Yamada *et al*., 2009; Taniguchi and Cousins, 2018; Kato *et al*., 2023). This
294 movement has also been observed in various C_4 plants (Kato *et al*., 2022). However, the
295 relationship between M chloroplast aggregative movement and adjacent BS cells has not yet been
296 confirmed. The analysis tracking individual M chloroplast movements toward adjacent BS cells
297 demonstrated that the initiation time of M chloroplast movement induced by blue light would not
298 be related to the distance from the BS cells at their initial position (Fig. 2B). Although M
299 chloroplasts initially located farther from the BS cells tended to move more toward the BS cells
300 (Fig. 3B-C), the efficiency of M chloroplast movement toward BS cells showed no correlation
301 with the initial distance from the BS cells (Fig. 3D). These results did not clarify whether BS cells
302 contributed to M chloroplast movement. To investigate the contribution of BS cells to the
303 aggregation of chloroplasts in M cells, we selectively removed the BS cell content by adjusting
304 the cutting angle and thickness (Fig. 4). The blue light-induced chloroplast aggregative movement
305 was less likely to occur in M cells in the absence of adjacent intact BS cells; rather, chloroplasts
306 showed a light avoidance response and were arranged parallel to the incident light direction (Fig.
307 5). This finding indicates that M chloroplast aggregative movement induced by blue light occurs
308 through communication with BS cells, unlike the generally known in chloroplast photo relocation
309 movement, which is a cell-autonomous response (Kagawa and Wada, 1994, 1996). Further
310 research is needed to identify the molecular nature of BS cell signals that affect M cells'
311 chloroplast movement. Our leaf-section imaging technique, compatible with fluorescent
312 molecular sensors, microbeam irradiation systems, and pulse amplitude-modulated fluorometry

313 (PAM), will become an indispensable tool for investigating how chloroplast aggregative
314 movement is involved in C_4 photosynthesis.
315
316 **Supplementary data**
317 Supplementary Figure S1. Schematic diagram of the live leaf-section imaging
318 Supplementary Figure S2. Temporal changes in the intracellular arrangement of chloroplasts in
319 the absence of blue light
320 Supplementary Figure S3. Temporal changes in the intracellular arrangement of chloroplasts
321 under blue light irradiation
322 Supplementary Figure S4. Temporal changes in the degree of chloroplast aggregative
323 arrangements under blue light irradiation
324 Supplementary Figure S5. Temporal changes in the intracellular arrangement of chloroplasts
325 under blue light irradiation and after cessation of the irradiation
326 Supplementary Figure S6. Disruption of BS cell by laser microbeam irradiation
327 Supplementary Movie S1. Live leaf-section imaging under continuous blue light irradiation from
328 the adaxial side of the leaf section
329 Supplementary Movie S2. Live leaf-section imaging under no blue light irradiation
330 Supplementary Movie S3. Live leaf-section imaging under blue-light irradiation and after
331 cessation of irradiation
332 Supplementary Movie S4. Live leaf-section imaging under continuous blue light irradiation from
333 the abaxial side of the leaf section
334 Supplementary Movie S5. Tracking of M chloroplast movement
335 Supplementary Movie S6. Live leaf-section imaging of M chloroplasts with adjacent BS cell
336 content under blue light irradiation from the adaxial side of the longitundinal leaf section
337 Supplementary Movie S7. Live leaf-section imaging of M chloroplasts without adjacent BS cells
338 under blue light irradiation from the adaxial side of the longitundinal leaf section

Acknowledgments

The author (Y.K.) would like to take this opportunity to thank the "Interdisciplinary Frontier Next-Generation Researcher Program of the Tokai Higher Education and Research System."

Author contributions

Y.K. M.T. Y.S. designed the study. Y.K. performed all the experiments, analyzed the data, and wrote the manuscript. M.T., T.O., and Y.S. supported the writing of the manuscript.

Conflict of Interest

No conflict of interest declared.

Funding Statement

This study was supported by the Japan Society for the Promotion of Science (JSPS KAKENHI). Grant Numbers: JP20H02966 and JP23H02195 (to M. T.). Grant: Number JP23K26799 (to Y. S.). Grant Number: JP- MJSP2125 (to Y. K.).

Data Availability Statement

For data available within the article or its supplementary materials: All data supporting the findings of this study are available within the paper and supplementary materials published online.

References

Brown WV. 1975. Variations in anatomy, associations, and origins of Kranz tissue. *American Journal of Botany* **62**, 395–402.

Dengler NG, Nelson T. 1999. Leaf structure and development in C_4 plants. In: Sage RF, ed. *C_4 Plant Biology*. San Diego: Academic Press, 133–172.

Haupt W, Scheuerlein R. 1990. Chloroplast movement. *Plant, Cell & Environment*, **13** 595-614.

Hatch MD. 1999. C_4 Photosynthesis: a historical overview. In: Sage RF, ed. *C_4 Plant Biology*. San Diego: Academic Press, 17–46.

Inoue Y, Shibata K. 1973. Light-induced chloroplast rearrangements and their action spectra as measured by absorption spectrophotometry. *Planta* **114**, 341‑358.

Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M. 2001. *Arabidopsis* NPL1: A Phototropin homolog controlling the chloroplast high-light avoidance response. *Science* **291**, 2138-2141.

Kagawa T, Wada M. 1994 Brief irradiation with red or blue light induces orientational movement of chloroplasts in dark-adapted prothallial cells of the fern *Adiantum*. *Journal of Plant Research* **107**, 389–398.

Kagawa T, Wada M. 1996. Phytochrome and blue-light-absorbing pigment-mediated directional movement of chloroplasts in dark-adapted prothallial cells of fern *Adiantum* as analyzed by microbeam irradiation. *Planta* **198**, 488–493.

Kato Y, Oi T, Taniguchi M. 2023. Aggregative movement of C_4 mesophyll chloroplasts is promoted by low CO_2 under high intensity blue light. *Plant Biology* **25**, 563–570.

Kato Y, Tsukaguchi T, Yata I, Yamamura R, Oi T, Taniguchi T. 2022. Aggregative movement of mesophyll chloroplasts occurs in a wide variety of C_4 plant species. *Flora* **294**, 152133.

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.

Lal A, Edwards EG. 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**, 403–415.

Maai E, Miyake H, Taniguchi M. 2011a. Differential positioning of chloroplasts in C_4 mesophyll and bundle sheath cells. *Plant Signaling and Behavior* **6**, 1111–1113.

Maai E, Nishimura K, Takisawa R, Nakazaki T. 2020. Diurnal changes in chloroplast positioning and photosynthetic traits of C_4 grass finger millet. *Plant Production Science* **23**, 477–489.

Maai E, Shimada S, Yamada M, Sugiyama T, Miyake H, Taniguchi M. 2011b. The avoidance and aggregative movements of mesophyll chloroplasts in C_4 monocots in response to blue light and abscisic acid. *Journal of Experimental Botany* **62**, 3213–3221.

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C_4 photosynthesis. *Annual Review of Plant Biology* **63**, 19–47.

Senn G. 1908. *Die Gestalts—und Lageveränderung der Pflanzen— Chromatophoren*. Wilhelm Engelmann, Leipzig.

Stata M, Sage TL, Rennie TD, Khoshravesh R, Sultmanis S, Khaikin Y, Ludwig M, Sage RF. 2014. Mesophyll cells of C_4 plants have fewer chloroplasts than those of closely related C_3 plants. *Plant Cell and Environment* **37**, 2587–2600.

Taniguchi M, Cousins AB. 2018. Significance of C_4 leaf structure at the tissue and cellular levels. In: Adams WW, Terashima I, ed. *Leaf: A Platform for Performing Photosynthesis*. Springer International Publishing AG. **44**, 255–279.

Wada M, Kadota A, Furuya M. 1983. Intracellular localization and dichroic orientation of phytochrome in plasma membrane and/or ectoplasm of a centrifuged protonema of fern *Adiantum capillus-veneris* L. *Plant and cell physiology*. ***24*,** 1441-1447.

Wada M, Kagawa T, Sato Y. 2003. Chloroplast movement. *Annual Review of Plant Biology* **54**, 455–468.

Wada M. 2013. Chloroplast movement. *Plant Science* **210**, 177–182.

Wada M. Kong SG. 2018. Actin-mediated movement of chloroplasts. *Journal of Cell Science* **131**, 210310.

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.

Table 1. M chloroplast motility for 1h.

Adaxial side	Blue light	No irradiation	Turn-off after 4 h blue light
Speed of chloroplast movement (μm min^{-1})	0.28±0.14 a	0.04±0.02 c	0.09±0.09 b
Displacement/Total movement	0.42±0.24 a	0.00±0.54 c	-0.52±0.66 b
Abaxial side			
Speed of chloroplast movement (μm min^{-1})	0.20±0.16 a	0.04±0.02 c	0.07±0.04 b
Displacement/Total movement	0.23±0.32 a	-0.31±0.45 b	-0.21±0.49 b
Both sides			
Speed of chloroplast movement (μm min^{-1})	0.24±0.15 a	0.04±0.02 b	0.08±0.14 b
Displacement/Total movement	0.35±0.28 a	-0.16±0.52 b	-0.38±0.60 b

Each value represents the mean ±SD. Each value was calculated 1 h after the initial irradiation in the blue light irradiation experiment, 1 h after the initial observation in the no blue light irradiation experiment, and 1 h after irradiation was turned off in the turn-off of blue light irradiation experiment. Different letters indicate a significant difference at $p < 0.05$ (Steel–Dwass test, adaxial side chloroplast numbers = 35-39. abaxial side chloroplast numbers = 33-36).

Table 2. M chloroplast motility for 1 h blue light irradiation with or without adjacent BS cell contents.

	With BS cell contents	**Without** BS cell contents	
Speed of chloroplast movement (μm min^{-1})	0.37±0.12	0.34±0.10	
Displacement/Total movement	0.41±0.22	0.29±0.23	*

Each value represents the mean (±SD). Asterisks indicate significant differences at $p < 0.05$ (Mann–Whitney *U* test, chloroplast number = 44).

Fig. 1. Continually observation changes in M chloroplast arrangement within the same leaf tissue in response to blue light

Images of transverse leaf tissue from finger millet leaf blades. The leaf section was irradiated with blue light (500 μmol m^{-2} s^{-1}) from the adaxial side. The upper part of each image shows the adaxial side. (A, B) Images were obtained after blue light irradiation for 0 and 4 h. (C) Comparison between 0 and 4 h after blue light irradiation. The images in (A) and (B) were binarized, and the areas that appeared and were lost after 4 h are shown in magenta and cyan, respectively. Areas that did not change are shown in gray. This image was created using the "Make Binary" and "Image Calculator – Subtract" functions in ImageJ. M, mesophyll cell; BS, bundle sheath cell; V, vascular bundle. Bars = 50 μm.

Fig. 2. Temporal change in the distance of each M chloroplast from BS cell with blue light irradiation

The leaf section was irradiated with blue light (500 μmol m^{-2} s^{-1}) from the adaxial side of the leaf section. (A) Images of adaxial M cells at the initial observation (−0.5 h) and 4 h after irradiation. A dot indicates the chloroplast position at each observation time, and chloroplast movement up to that time is indicated by a line. (B) Temporal changes in linear distance from BS cells in each chloroplast at each time were plotted every minute from 0.5 h before to 4 h after the blue light irradiation. If the chloroplasts could not be tracked because they overlapped or became out of focus, tracking was terminated at that point. Red lines represent chloroplasts that moved closer to the BS cells, and blue lines represent those that moved farther from the BS cells after 4 h of blue light irradiation compared to their initial position at the initial stage of irradiation (t = 0). The line colors were determined based on the tracking endpoint for chloroplasts that could not be tracked until 4 h of blue light irradiation. More than ten chloroplasts were randomly selected for monitoring from the three individuals' adaxial side of the leaf sections (chloroplast number = 39). M: mesophyll cells; BS: bundle sheath cells. Bars in (A) = 25 μm.

Figure 3. Correlation between the distance of M chloroplast from BS cell and motility of abaxial M chloroplast

(A) Schematic diagram showing M chloroplast positions before and after blue light irradiation and its distances traveled during the time. The total distance a chloroplast moved during the first hour of blue light irradiation is "*Total movement,*" and the linear distance to the BS cell side that the chloroplast moved for 1 h is "*Displacement*." The linear distance between the chloroplast and the BS cell at the initial blue light irradiation is defined as the "*Initial position.*" (B) Correlations

between *Initial position* and *Total movement*. (C) Correlations between *Initial Position* and *Displacement*. Negative *Displacement* values indicate that the chloroplast moved in the opposite direction from the BS cell side during the 1 h irradiation period. (D) Correlations between *Initial position* and *Displacement*/*Total movement*. *Displacement*/*Total movement* indicates migration efficiency to BS cells; the closer to +1, the more chloroplast movement is directed toward the BS cell side. The analysis was based on the data on chloroplast behavior shown in Fig. 2C (chloroplast number = 39). Pearson's correlation test was conducted to evaluate the relationship, with the null hypothesis assuming no correlation between the two values ($p < 0.05$). The correlation coefficient was shown as r in each graph when a significant correlation was found.

Fig. 4. Sectioning of leaf tissue and removal of BS cell contents

(A) Schematic diagram of the leaf blade of finger millet. A transverse section was made by cutting the leaf blade perpendicular to the vascular bundle (blue dash line), and a longitudinal section was cut parallel to the vascular bundle (red dash line). Microscope images of a transverse section (B) and a longitudinal section (C). Since BS cells are larger than M cells, it is possible to cut only BS cells while leaving M cells intact, as shown by the red dashed line in the microscopic image (B). (D, E) adaxil side of longitudinal sections. (D) The longitudinal section with intact BS cells was obtained by sectioning at 60 µm thickness. (E) The longitudinal section without BS cell contents was obtained by sectioning at 40 µm thickness. M, mesophyll cell; BS, bundle sheath cell; V, vascular bundle. Bars in (B–E) = 50 µm.

Fig. 5. Comparison of adaxial M chloroplast movements with and without BS cell contents

Longitudinal sections with BS cell contents (A) or without the contents (B) were irradiated with blue light (500 µmol m^{-2} s^{-1}) from the adaxial side. After 4 h of blue light irradiation, irradiation was stopped. The upper part of each image shows the adaxial side. Bars = 25 µm in (A, B). (C) Violin plots of M chloroplast relative distance (RD) from BS cells 0 and 4 h after blue light irradiation. Chloroplasts from three M cells per individual were measured, and three individuals were analyzed for each treatment (chloroplast number in each individual = 38–74). The width of each violin plot represents the probability density, and the red points in the violin plots indicate the *RD* median of each individual. Asterisks (*) indicate significant differences in the mean *RD* medians around each vascular bundle with and without BS cells ($p < 0.05$, Student's t-test, $n = 3$).

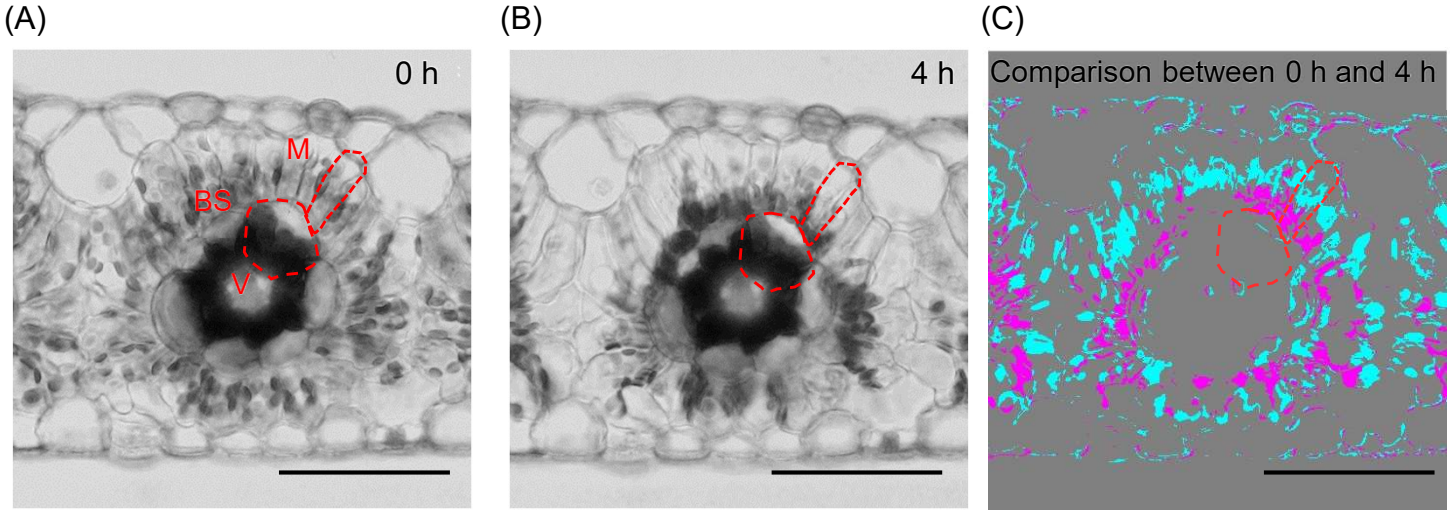

Fig. 1. Continually observation changes in M chloroplast arrangement within the same leaf tissue in response to blue light

Images of transverse leaf tissue from finger millet leaf blades. The leaf section was irradiated with blue light (500 μmol m^{-2} s^{-1}) from the adaxial side. The upper part of each image shows the adaxial side. (A, B) Images were obtained after blue light irradiation for 0 and 4 h. (C) Comparison between 0 and 4 h after blue light irradiation. The images in (A) and (B) were binarized, and the areas that appeared and were lost after 4 h are shown in magenta and cyan, respectively. Areas that did not change are shown in gray. This image was created using the "Make Binary" and "Image Calculator – Subtract" functions in ImageJ. M, mesophyll cell; BS, bundle sheath cell; V, vascular bundle. Bars = 50 μm.

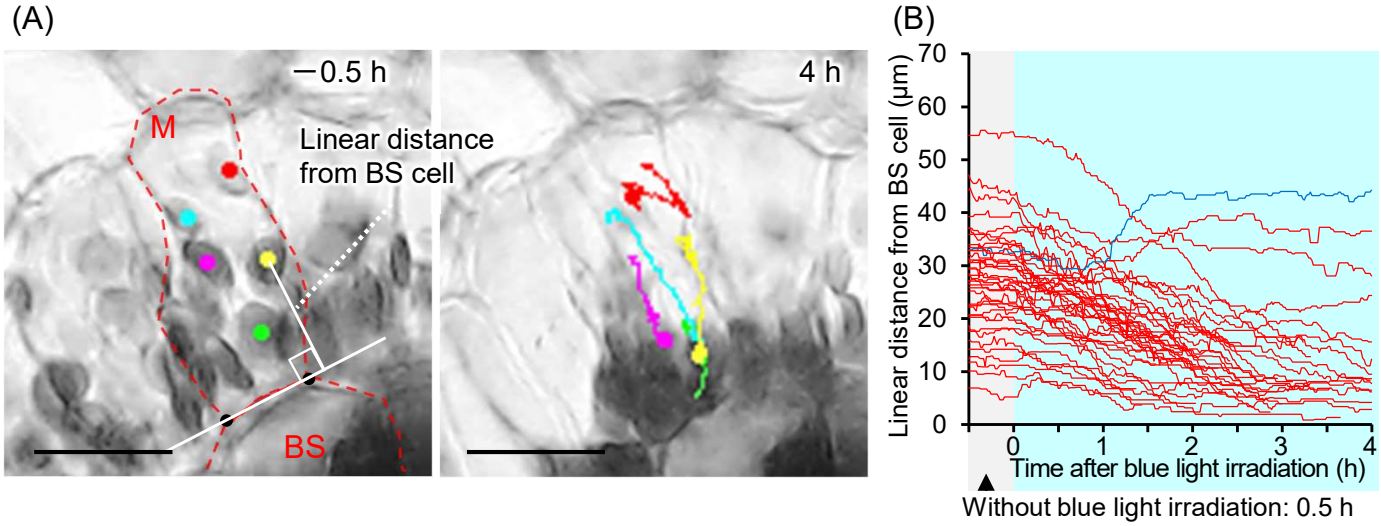

Fig. 2. Temporal change in the distance of each M chloroplast from BS cell with blue light irradiation

The leaf section was irradiated with blue light (500 μmol m^{-2} s^{-1}) from the adaxial side of the leaf section. (A) Images of adaxial M cells at the initial observation (−0.5 h) and 4 h after irradiation. A dot indicates the chloroplast position at each observation time, and chloroplast movement up to that time is indicated by a line. (B) Temporal changes in linear distance from BS cells in each chloroplast at each time were plotted every minute from 0.5 h before to 4 h after the blue light irradiation. If the chloroplasts could not be tracked because they overlapped or became out of focus, tracking was terminated at that point. Red lines represent chloroplasts that moved closer to the BS cells, and blue lines represent those that moved farther from the BS cells after 4 h of blue light irradiation compared to their initial position at the initial stage of irradiation (t = 0). The line colors were determined based on the tracking endpoint for chloroplasts that could not be tracked until 4 h of blue light irradiation. More than ten chloroplasts were randomly selected for monitoring from the three individuals' adaxial side of the leaf sections (chloroplast number = 39). M: mesophyll cells; BS: bundle sheath cells. Bars in (A) = 25 μm.

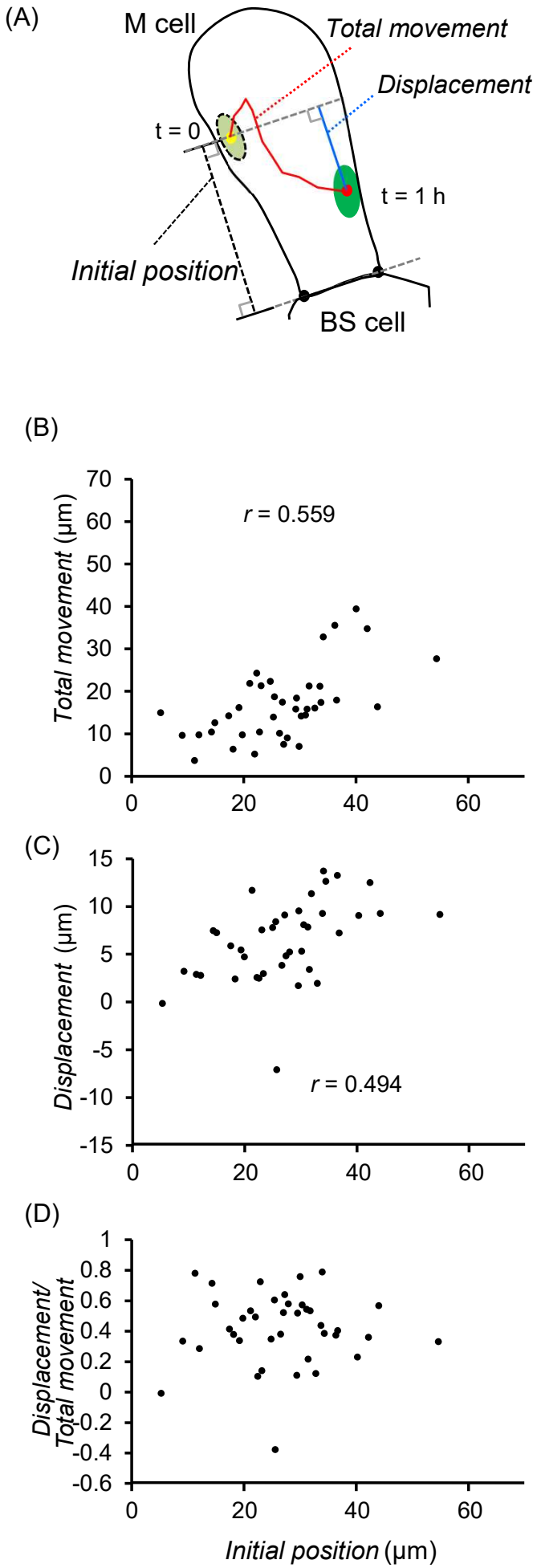

Figure 3. Correlation between the distance of M chloroplast from BS cell and motility of abaxial M chloroplast

(A) Schematic diagram showing M chloroplast positions before and after blue light irradiation and its distances traveled during the time. The total distance a chloroplast moved during the first hour of blue light irradiation is "*Total movement,*" and the linear distance to the BS cell side that the chloroplast moved for 1 h is "*Displacement*." The linear distance between the chloroplast and the BS cell at the initial blue light irradiation is defined as the "*Initial position.*" (B) Correlations between *Initial position* and *Total movement*. (C) Correlations between *Initial Position* and *Displacement*. Negative *Displacement* values indicate that the chloroplast moved in the opposite direction from the BS cell side during the 1 h irradiation period. (D) Correlations between *Initial position* and *Displacement*/*Total movement*. *Displacement*/*Total movement* indicates migration efficiency to BS cells; the closer to +1, the more chloroplast movement is directed toward the BS cell side. The analysis was based on the data on chloroplast behavior shown in Fig. 2C (chloroplast number = 39). Pearson's correlation test was conducted to evaluate the relationship, with the null hypothesis assuming no correlation between the two values ($p < 0.05$). The correlation coefficient was shown as r in each graph when a significant correlation was found.

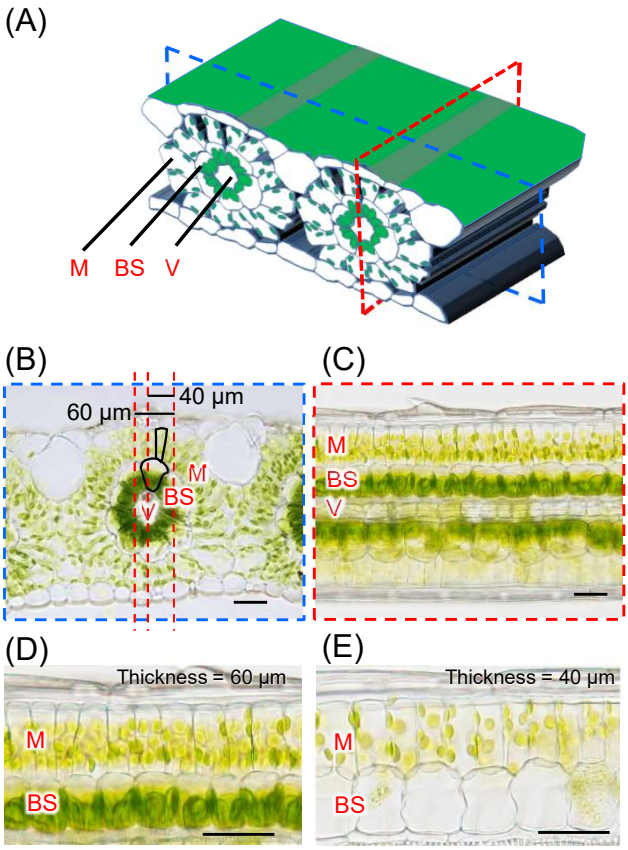

Fig. 4. Sectioning of leaf tissue and removal of BS cell contents

(A) Schematic diagram of the leaf blade of finger millet. A transverse section was made by cutting the leaf blade perpendicular to the vascular bundle (blue dash line), and a longitudinal section was cut parallel to the vascular bundle (red dash line). Microscope images of a transverse section (B) and a longitudinal section (C). Since BS cells are larger than M cells, it is possible to cut only BS cells while leaving M cells intact, as shown by the red dashed line in the microscopic image (B). (D, E) adaxil side of longitudinal sections. (D) The longitudinal section with intact BS cells was obtained by sectioning at 60 µm thickness. (E) The longitudinal section without BS cell contents was obtained by sectioning at 40 µm thickness. M, mesophyll cell; BS, bundle sheath cell; V, vascular bundle. Bars in (B–E) = 50 µm.

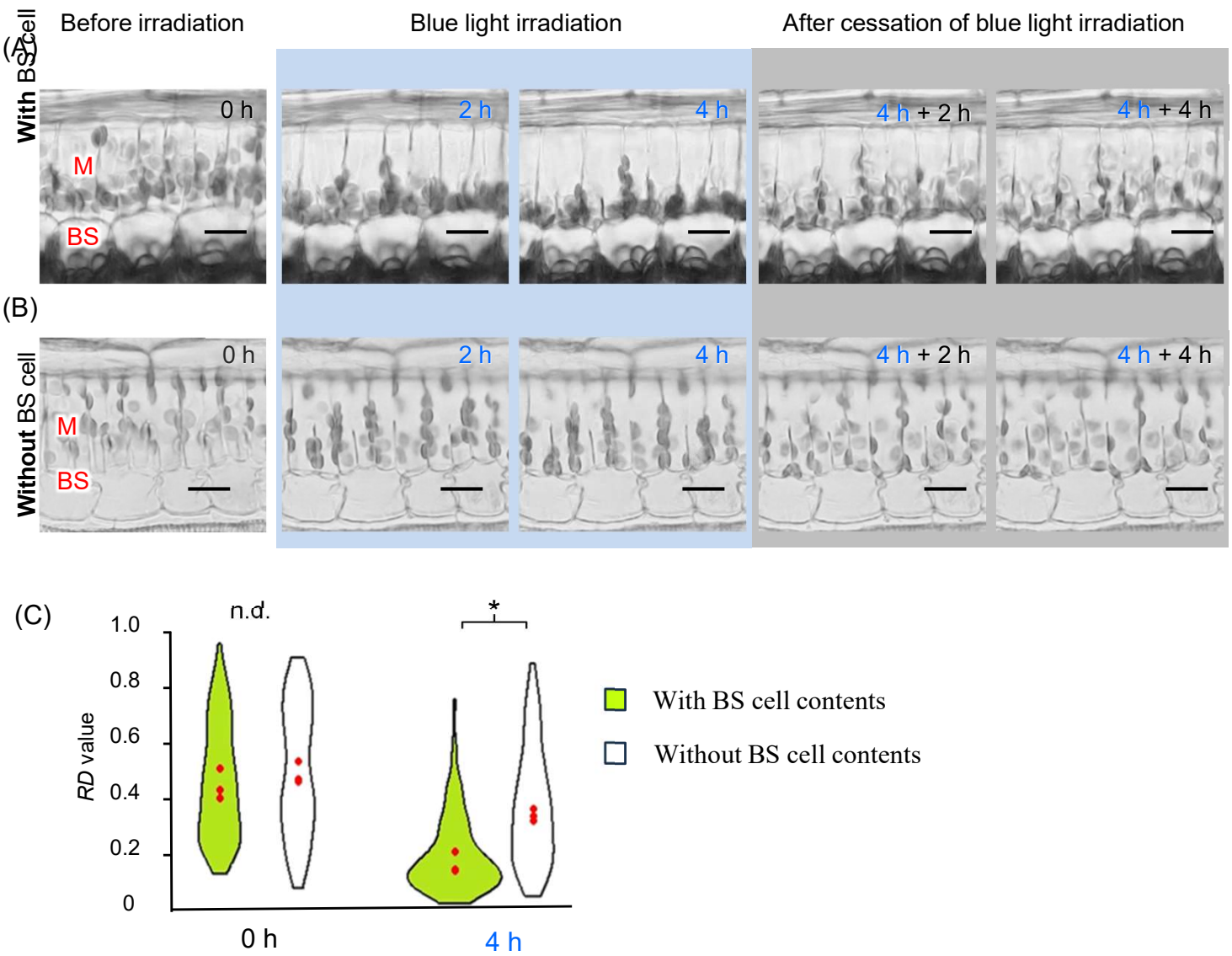

Fig. 5. Comparison of adaxial M chloroplast movements with and without BS cell contents

Longitudinal sections with BS cell contents (A) or without the contents (B) were irradiated with blue light (500 μmol m^{-2} s^{-1}) from the adaxial side. After 4 h of blue light irradiation, irradiation was stopped. The upper part of each image shows the adaxial side. Bars = 25 μm in (A, B). (C) Violin plots of M chloroplast relative distance (RD) from BS cells 0 and 4 h after blue light irradiation. Chloroplasts from three M cells per individual were measured, and three individuals were analyzed for each treatment (chloroplast number in each individual = 38–74). The width of each violin plot represents the probability density, and the red points in the violin plots indicate the *RD* median of each individual. Asterisks (*) indicate significant differences in the mean *RD* medians around each vascular bundle with and without BS cells ($p < 0.05$, Student's *t*-test, $n = 3$).