Chloroplasts are plant organelles that function as factories that convert light energy into chemical energy through photosynthesis to support plants’ life. Chloroplasts change their intracellular positions in response to environmental light conditions (Wada et al. 2003, Gabrys 2004, Suetsugu and Wada 2007a, Suetsugu and Wada 2007b, Suetsugu and Wada 2009). In the dark, chloroplasts are located along the anticlinal walls and/or on the bottom of cells; however, when transferred to weak or moderate light, they relocate along periclinal walls for efficient light energy capture (accumulation response). In contrast, they move away from strong light and relocate along anticlinal walls to avoid photodamage (avoidance response) (Kasahara et al. 2002). In Arabidopsis thaliana, these chloroplast photomovements are mediated by the blue light receptors phototropin 1 (phot1) and phototropin 2 (phot2) (Jarillo et al. 2001, Kagawa et al. 2001, Sakai et al. 2001). The accumulation movements are mediated by both phot1 and phot2, whereas the avoidance movements are mediated by phot2 alone (Suetsugu and Wada 2007a, Suetsugu and Wada 2007b). Recently, a small contribution of phot1 to the avoidance response has been suggested (Luesse et al. 2010).

Cytoskeletal components, such as actin filaments and microtubules, are intimately involved in the regulation of organelle movement and positioning. In plant cells, organelle movement and distribution are mainly mediated by the actin cytoskeleton (Williamson 1993, Shimmen and Yokota 2004, Staiger and Blanchon 2006, Shimmen 2007, Blanchon et al. 2010, Ueda et al. 2010). It has been suggested that actin filaments play an important role in chloroplast movement (Wada et al. 2003, Gabrys 2004, Suetsugu and Wada 2007a, Suetsugu and Wada 2007b, Suetsugu and Wada 2009, Takagi et al. 2009, Luesse et al. 2010).

**Introduction**

The phototropin (phot)-dependent intracellular relocation of chloroplasts is a ubiquitous phenomenon in plants. We have previously revealed the involvement of a short cp-actin (chloroplast actin) filament-based mechanism in this movement. Here, the reorganization of cp-actin filaments during the avoidance movement of chloroplasts was analyzed in higher time resolution under blue GFP (green fluorescent protein) excitation light in an actin filament-visualized line of Arabidopsis thaliana. Under standard background red light of 89 μmol m⁻² s⁻¹, cp-actin filaments transiently disappeared at approximately 30 s and reappeared in a biased configuration on chloroplasts approximately 70 s after blue excitation light irradiation. The timing of biased cp-actin reappearance was delayed under the background of strong red light or in the absence of red light. Consistently, chloroplast movement was delayed under these conditions. In phot1 mutants, acceleration of both the disappearance and reappearance of cp-actin filaments occurred, indicating an inhibitory action of phot1 on reorganization of cp-actin filaments. Avoidance movements began sooner in phot1 than in wild-type plants. No reorganization of cp-actin filaments was seen in phot2 or phot1phot2 mutants lacking phot2, which is responsible for avoidance movements. Surprisingly, jac1 (j-domain protein required for chloroplast accumulation response 1) mutants, lacking the accumulation response, showed no avoidance movements under the whole-cell irradiation condition for GFP observation. Cp-actin filaments in jac1 did not show a biased distribution, with a small or almost no transient decrease in the number. These results indicate a close association between the biased distribution of cp-actin filaments and chloroplast movement. Further, JAC1 is suggested to function in the biased cp-actin filament distribution by regulating their appearance and disappearance.

**Keywords:** Actin filament • Arabidopsis • Chloroplast movement • Photomovement • Phototropin • Phytochrome.

**Abbreviations:** CHUP1, chloroplast unusual positioning 1; cp-actin filament, chloroplast actin filament; GFP, green fluorescent protein; JAC1, J-domain protein required for chloroplast accumulation response 1; KAC1 and KAC2, kinesin-like protein for actin-based chloroplast movement 1 and 2; phot, phototropin

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Suetsugu et al. 2010a), although no clear evidence had been shown to support this. Recently, we analyzed actin reorganization during chloroplast movement induced by partial cell irradiation in an actin filament-visualized line of A. thaliana stably expressing green fluorescent protein (GFP)–mTalin and found a unique structure made of short actin filaments between chloroplasts and the plasma membrane. We named this structure ‘chloroplast actin filaments’ (cp-actin filaments) (Kadota et al. 2009, Suetsugu et al. 2010b). When accumulation or avoidance movements were induced, high and low numbers of cp-actin filaments on the front and rear regions of the moving chloroplast, respectively, were established. The speed of chloroplast movements correlated with the difference in amounts of cp-actin between the front and the rear ends of chloroplasts. Further, mutants lacking cp-actin filaments did not show chloroplast photomovement, revealing that the cp-actin filaments are involved in the mechanism of chloroplast photorelocation (Kadota et al. 2009, Suetsugu et al. 2010b).

In contrast, cytoplasmic actin filaments were suggested to have no apparent role in the movement. Cp-actin filaments were also found in the evolutionarily distant cells of the moss Physcomitrella patens (Yamashita et al. 2011).

In the above studies, the time interval between fluorescence acquisitions had to be longer than 5 or 10 min because blue GFP excitation light is also absorbed by phots. This would reduce the light absorption gradient in the cell, which is necessary for the avoidance and accumulation responses, even though partial cell irradiation with a microbeam was applied for the induction of chloroplast photorelocation. However, in these studies we also found that continuous irradiation of whole cells with the blue GFP excitation light induced chloroplast avoidance movements, indicating that the time interval between fluorescence acquisitions could be reduced to as short as 3 s. In this work, we further analyzed, with higher time resolution, the details of cp-actin filament reorganization in wild-type and chloroplast photomovement-deficient mutant plants.

**Results and Discussion**

**Reorganization of cp-actin filaments during avoidance movement induced by continuous irradiation with blue GFP excitation light**

To examine the reorganization of cp-actin filaments on chloroplasts, thin sections were prepared from the petiole of a mature leaf of transgenic Arabidopsis plants expressing GFP–mTalin. Cells were continuously irradiated with blue GFP excitation light (estimated to be approximately 7,150 μmol m⁻² s⁻¹) under a fluorescence microscope. Background red light of 89 μmol m⁻² s⁻¹ was present continuously except during fluorescence image acquisition. GFP and Chl fluorescence were recorded at intervals of 5 s for 5 min (Fig. 1A, Supplementary Movie 1). The blue excitation light was set as low as possible to avoid bleaching while capturing the fluorescence images with a good signal to noise ratio. However, the light intensity was still strong enough to induce the avoidance response.

Observations of the dynamics of cp-actin filaments revealed that they disappeared transiently at approximately 30 s and then reappeared in a biased distribution on chloroplasts approximately 70 s after irradiation. At this time point, chloroplasts had just started to move toward the direction of cp-actin accumulation (Fig. 1B, Supplementary Movie 2). Occasionally, cp-actin filaments reappeared at several peripheral areas of a chloroplast but were gradually confined to one area (Fig. 1C, Supplementary Movie 3). In these cases, the distances chloroplasts traveled over 5 min were noticeably short (compare the movement paths in the far right panels of Fig. 1B and C). Although we attempted to determine whether the chloroplast movement or the formation of biased cp-actin filaments started first, the time resolution was not high enough to answer this question conclusively.

**Modulation of reorganization of cp-actin filaments by background red light during avoidance movements**

In the above experiments, as well as in our previous studies (Kadota et al. 2009, Suetsugu et al. 2010b), we used red light of approximately 89 μmol m⁻² s⁻¹ for background illumination as a standard condition. Because red light was reported to modulate the blue light-induced chloroplast relocation movement (Kagawa and Wada 2000, DeBlasio et al. 2003, Luesse et al. 2010), the red light effect on the reorganization of cp-actin filaments during the avoidance movement was examined. Actin reorganization was recorded every 3 s under various background light conditions, namely standard red light (89 μmol m⁻² s⁻¹), strong red light (570 μmol m⁻² s⁻¹) or no red light irradiation (Fig. 2, Supplementary Movies 4–6).

In all of the background light conditions, chloroplast movement correlated with a biased distribution of cp-actin filaments was established. In the background condition of 89 μmol m⁻² s⁻¹ standard red light and in the absence of red light, cp-actin filaments disappeared approximately 30 s after blue light irradiation (Table 1). Under strong red light, reduction in the number of cp-actin filaments was observed at approximately 30 s but they did not completely disappear. In the standard background red light, the timing of the biased reappearance of cp-actin filaments was slightly faster than that under the condition without red light (Table 1). Under a strong red light background, the biased distribution of cp-actin filaments on chloroplasts was gradually achieved after an initial delay (Table 1). When chloroplast movement under the three background conditions was analyzed as the distance traveled every 30 s, it was apparent that chloroplasts began moving in the order of standard red light, no red light and strong red light (Fig. 3A). Integrated distances traveled over 5 min were consistently shorter in the strong and no red light background (Fig. 3B). These results indicate that red light has both promotive and inhibitory effects on the avoidance movements,
depending on light intensity. Hence, it is suggested that red light modulates chloroplast photorelocation through the regulation of cp-actin reorganization.

Promotion of chloroplast accumulation movement by background red light was reported in Arabidopsis leaf mesophyll cells, and a possible contribution of red light enhancement to cytoplasmic motility was suggested (Kagawa and Wada 2000). Both promotion of the accumulation response and inhibition of the avoidance response by red light through phytochromes were reported by DeBlasio et al. (2003), and phytochrome B involvement in the modulation has been documented (Luesse et al. 2010). In the present study focusing on the avoidance movement, both promotive and inhibitory effects of red light were found on the blue light-induced reorganization of cp-actin filaments, which correlated well with the start of migration. This evidence indicates the possibility that red light modulates chloroplast movement through the regulation of cp-actin reorganization. The red light receptor(s) could be
phytochrome(s) as suggested in the above references, but the contribution of photosynthesis could not be disregarded since cooperative control of cytoplasmic motility by phytochrome and photosynthetic pigments is reported (Takagi et al. 1990, Dong et al. 1995). Further, photosynthesis-dependent chloroplast photorelocation was recently revealed in fern prothallial cells (Sugiyama and Kadota 2011).

Reorganization of cp-actin filaments in photomovement mutants

We next analyzed chloroplast movement and cp-actin reorganization in the photoreceptor mutants phot1, phot2 and phot1-phot2 and in jac1 (J-domain protein required for chloroplast accumulation response 1), which is reported to have a defect in chloroplast photorelocation. We next analyzed chloroplast movement and cp-actin reorganization in the photoreceptor mutants phot1, phot2 and phot1-phot2 and in jac1 (J-domain protein required for chloroplast accumulation response 1), which is reported to have a defect in chloroplast photorelocation.
specific to the weak light-induced accumulation movement of chloroplasts (Suetsugu et al. 2005) (Fig. 4).

In phot1 mutant plants, the same reorganization of cp-actin filaments and avoidance movement as in wild-type plants were observed (Fig. 4A, Supplementary Movie 7). However, both the timings in transient disappearance (20.3 ± 4.8 s) and biased reappearance of cp-actin filaments (38.4 ± 9.1 s) in phot1 mutants were faster than those in wild-type plants (Table 1). Time between disappearance and biased reappearance was calculated to be 18.2 ± 10.1 s, which is apparently shorter than that of the wild type. The mean values with standard deviations were obtained from 11 chloroplasts in four independent experiments. The values are significantly different from those of wild-type plants (Table 1) as determined by the t-test (P < 0.05). The time course analysis of chloroplast movement in phot1 mutants clearly indicates that the chloroplasts began moving earlier than those of wild-type plants (Fig. 5). Thus, phot1 inhibits avoidance movements probably through regulating cp-actin reorganization.

phot2 mutants are defective in avoidance movements, as reported in Kagawa et al. (2001). Consistently, in both phot2 and phot1phot2 mutants, no apparent change in cp-actin filament organization was observed, whereas the filaments themselves were highly dynamic (Fig. 4B, C, Supplementary Movies 8, 9). While phot2 chloroplasts showed accumulation movement under microbeam irradiation of a cell with strong blue light (Kadota et al. 2009), they showed no movement under the present light condition. phot1phot2 chloroplasts were also stationary during the observation periods. When chloroplast movements in the phot1, phot2 and phot1phot2 mutant plants were analyzed as the integrated distance traveled over 5 min, it was apparent that phot2 and phot1phot2 chloroplasts showed almost no movement but that phot1 chloroplasts showed longer movements than those of wild-type plants (Fig. 6). Therefore, the avoidance movements and reorganization of cp-actin filaments correlated well.

jac1 was isolated as a mutant specifically lacking the weak light-induced accumulation movement of chloroplasts but having nearly normal avoidance movements under strong blue light (Suetsugu et al. 2005). Surprisingly, however, the cp-actin reorganization during the chloroplast avoidance response was not observed in jac1 mutants. Even under continuous blue excitation light, neither the disappearance nor the biased relocation of cp-actin filaments occurred, with a small transient reduction if any (Fig. 4D, Supplementary Movie 10). Chloroplasts showed no movement and were stationary during the observation periods, as in phot2 and phot1phot2 mutants (Fig. 6). This jac1-1 phenotype was also confirmed in another allelic mutant, jac1-2 (data not shown).

Chloroplast photorelocation movements induced by partial cell irradiation with a microbeam in jac1

As jac1 was defective in the avoidance response under whole cell irradiation with blue excitation light for GFP, we conducted experiments on chloroplast relocation movements using partial cell irradiation with a microbeam 30 μm in width. As reported in Suetsugu et al. (2005), jac1-1 chloroplasts did not show accumulation movements under a blue microbeam of 3.8 μmol m⁻² s⁻¹. Consistently, no change in the organization of
Reorganization of cp-actin filaments under continuous blue excitation light in various mutants. Reorganization of cp-actin filaments under continuous blue excitation light was analyzed in phot1 (A), phot2 (B), phot1phot2 (C) and jac1-1 (D) mutant plants under the background red light of 89 μmol m⁻² s⁻¹. Note that no particular reorganization of cp-actin filaments was evident in any of the mutants except phot1. phot1 mutant plants showed a reorganization similar to that of wild-type plants, but the timing of the reorganization in phot1 mutant plants was earlier than that in wild-type plants. Orange dotted lines indicate the rear edge of chloroplasts.

Fig. 5 Changes in chloroplast speed during avoidance movements under continuous excitation blue light in the wild type and phot1 mutants. The speed of chloroplast movement was determined as the distance traveled every 30 s for 5 min. Bars indicate the standard deviation. The number of chloroplasts examined in phot1 was 11 derived from four independent experiments. Wild-type data are the same as in Fig. 3A.

Fig. 6 Chloroplast movement during the avoidance response under continuous blue excitation light in the wild type, and in phot1, phot2, phot1phot2 and jac1-1 mutants. Chloroplast movement was determined as the integrated distance traveled over 5 min. Bars indicate the standard deviation. The number of chloroplasts examined in each mutant was 11–17 derived from 3–4 independent experiments. *Values significantly different from that of the wild type as determined by t-test (P < 0.05).
cp-actin filaments was observed (Supplementary Movies 11, 12). A very few chloroplasts in jac1-1 mutants showed an avoidance movement under the weak blue microbeam irradiation as reported by Suetsugu et al. (2005). In the chloroplasts showing avoidance movements, biased distribution of cp-actin filaments was obvious (encircled chloroplast in Supplementary Movie 12). Under a blue microbeam of 377 μmol m⁻² s⁻¹, chloroplasts around the beam edge showed normal avoidance movements, and the biased localization of cp-actin filaments was observed on the moving chloroplasts in jac1-1 mutants (Fig. 7B-c, Supplementary Movie 14) as in wild-type plants (Fig. 7B-a, Supplementary Movie 13).

In the wild type, the chloroplasts located inside the beam area showed the disappearance of cp-actin filaments (Fig. 7B-b), leading to their increased motility in random directions (Fig. 8A) as reported in Kadota et al. (2009). However, cp-actin filaments in jac1-1 were distributed all around the chloroplast periphery (Fig. 7B-d). Chloroplast motility filaments was observed on the moving chloroplasts in jac1-1 mutants.
was clearly lower than that of wild-type plants (Fig. 8A). These results indicate that jac1 mutants also have a defect in the responses to strong blue light. Under conditions of weak light, chloroplast motility in jac1-1 mutants is not as different but is slightly lower than that in wild-type plants (Fig. 8B). Thus, the results indicate that JAC1 acts in the regulation of cp-actin disappearance and biased appearance downstream of both phot1 and phot2. Further, because the chloroplast avoidance response in jac1 mutants was evident under microbeam irradiation but not under whole-cell irradiation with strong GFP excitation light, whereas the intracellular light absorption gradient of phot2 was apparently less steep in the latter condition, it is also possible that JAC1 acts in the regulation of sensitivity to the light absorption gradient.

In the present study, cp-actin reorganization during avoidance movements under strong GFP excitation light was analyzed (Fig. 9). Under various conditions, the avoidance movements of chloroplasts and the cp-actin filament organization correlated quite well. Background red light appeared to affect chloroplast movement through the regulation of cp-actin reorganization, depending on the light intensity. Reorganization steps were accelerated in the phot1 mutants, indicating an inhibitory action of phot1 on the avoidance response. There is physiological and genetic evidence that the signaling pathway for the accumulation response (regulated mainly by phot1 in A. thaliana) is activated even under the strong light condition and that the avoidance and accumulation responses compete with each other (Suetsugu and Wada 2009, Kodama et al. 2010). Thus, attenuation of the accumulation response may cause the enhanced avoidance movements in the phot1 mutant plants.

Fig. 8 Motility of chloroplasts in the beam area under microbeam blue light in the wild type and jac1-1 mutants. The motility of chloroplasts in the beam area was determined as the mean and standard deviation of the distance traveled over 10 min under 377 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (A) and 3.8 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (B) blue light. The number of chloroplasts examined in each condition was 17–21 derived from 5–10 independent experiments. *Values significantly different from that of the wild type as determined by t-test (\( P < 0.05 \)).

![Fig. 9 Scheme showing the reorganization of cp-actin filaments during chloroplast avoidance movements. The average time of each event occurring after the induction of avoidance movements is shown below the chloroplast. Factors regulating the reorganization of cp-actin filaments are also shown. CHUP1, chloroplast unusual positioning 1 (Kadota et al. 2009); JAC1, J-domain protein required for chloroplast accumulation response 1 (present work); KAC1 and KAC2, kinesin-like protein for actin-based chloroplast movement 1 and 2 (Suetsugu et al. 2010b); phot1, phototropin 1 (present work); phot2, phototropin 2 (present work and Kadota et al. 2009); PMI2, plastid movement impaired 2 (Kodama et al. 2010); WEB1, weak chloroplast movement under blue light 1 (Kodama et al. 2010).

Several proteins have been identified by the analyses of mutants with defects in chloroplast photorelocation movements (Fig. 9). In chup1 mutant plants, cp-actin filaments are missing, while cytoplasmic actin filaments are present (Kadota et al. 2009), suggesting the crucial role of CHUP1 (chloroplast...
unusual positioning 1) in the generation of cp-actin filaments. CHUP1 is a chloroplast outer envelope protein with a proline-rich domain and an actin-binding domain, implicating its actin polymerization activity (Oikawa et al. 2003, Oikawa et al. 2008, Schmidt von Braun and Schleiff 2008). CHUP1 is suggested to reside as a dimer on the chloroplast, where intramolecular structure formation might result in the close proximity of the two domains (Lehmann et al. 2011). Kinesin-like proteins KAC1 and KAC2, in which no microtubule-binding activity could be detected, seem likely to work redundantly and, when both genes are mutated, the cp-actin system is lost (Suetsugu et al. 2010b).

Unexpectedly, the avoidance response and cp-actin filament reorganization were impaired in the jac1 mutant depending on the light conditions. Although it is thought that jac1 is deficient specifically in the accumulation response (Suetsugu et al. 2005), a recent study (Kodama et al. 2010) as well as the present study revealed that the avoidance response is aberrant in the jac1 mutant. Hence, these results indicate that JAC1 has another function in addition to its essential function in the accumulation response. Recently, a similar phenotype to jac1 on the cp-actin system was found in web1 (Kodama et al. 2010) and pmii2 (Luesse et al. 2006, Kodama et al. 2010), which show weak chloroplast movement in both the accumulation and avoidance responses. Kodama et al. (2010) performed partial cell irradiation experiments with a microbeam in web1 and pmii2 mutants and reported that cp-actin filaments did not disappear under strong light but showed biased relocalization in moving chloroplasts. Furthermore, genetic analysis suggested that WEB1 and PMI2 mediate the avoidance movement by modulating JAC1 activity (Kodama et al. 2010). Therefore, it is possible that, at least under certain conditions, JAC1 and WEB1/PMI2 cooperatively regulate cp-actin filament reorganization during the avoidance response. Further, very recently, Whippo et al. (2011) identified thrumin1 as a light-regulated actin-binding protein that is likely to co-localize with cytoplasmic actin filaments and bundle them upon blue light irradiation. While cp-actin filaments were not observed in the study, it would be very interesting to know its relationship to the cp-actin filaments, because the protein links blue light absorption to the reorganization of actin filaments.

Materials and Methods

Plant materials and culture conditions

Seeds of A. thaliana expressing GFP–mTalin in the wild type (Col g/l), and in phot1-5, phot2-1, phot1-5phot2-1, jac1-1 and jac1-2 mutant backgrounds were grown in plastic dishes or pots under light/dark (16 h/8 h) conditions at 23 °C as described previously (Kadota et al. 2009). Light was provided by three white fluorescent tubes (FL15W; Toshiba Lighting and Technology Corp.). Inner cells of petioles from fully expanded leaves were used for experiments. Petioles were harvested from 4-week-old seedlings and immersed in silicon oil (KF-96-20CS or -50CS; Shin-Etsu Chemical Co., Ltd.). They were hand-sectioned, and the thin sections were mounted with silicon oil. They were incubated overnight under weak white light before the experiments.

Fluorescence microscopy and light treatment

Cp-actin filament dynamics were observed under fluorescence microscopy in the same way as described previously (Kadota et al. 2009). Briefly, GFP and Chl fluorescence were captured with Zeiss XF 116-2 and FS17 (the band-pass filter BP515–565 was replaced with the long-pass filter LP520) filter units, respectively, using a plan apochromat ×63 objective (NA 1.4, Carl Zeiss). Blue GFP excitation light (estimated to be approximately 7,150 μm ol−1 m−2 s−1 (1,900 W m−2)) was continuously present for 5 min, during which fluorescence images were acquired at intervals of 3 or 5 s. Background red light was also present continuously during this period, except during fluorescence image acquisition. The red light was obtained by inserting an interference filter (662.2 nm peak wavelength, 5 nm half-band width, Vacuum Optics of Japan) in the transmission light path of the microscope. Fluorescence image acquisition during partial irradiation of a cell with a blue light microbeam was conducted using an epi-microbeam irradiation system as described previously (Kadota et al. 2009, Yamashita et al. 2011). Blue light for the microbeam was obtained by filtering light from a 100 W halogen lamp through an interference filter (451 nm peak wavelength, 32 nm half-band width, Vacuum Optics of Japan). Fluorescence images were captured with a cooled CCD camera (CoolSNAP HQ, Nippon Roper Co.).

Image analyses

For processing and analyses of fluorescence images, ImageJ (http://rsb.info.nih.gov/ij/) and Object Image (http://simon.bio.uva.nl/Object-Image/object-image.html) were used. For determination of time points for cp-actin disappearance and biased reappearance, image stacks of cp-actin filaments at 3 s interval for 5 min under blue GFP excitation light were inspected for their clarity (namely, a good signal to noise ratio) and for no change in the focus plane during the period. After sharpening by default setting of Object Image and manual contrast enhancement on ImageJ, the image stack file was played as a movie. The time point for transient disappearance of cp-actin filaments was determined in each chloroplast as the earliest time when short cp-actin filaments became lost or diminished from the whole area of a chloroplast. The time point for biased cp-actin reappearance was also determined in each chloroplast as the time when the sign of localized accumulation of short cp-actin filaments was recognized at the chloroplast periphery.
Cp-actin filaments can be distinguished from those of the cytoplasm by their lengths.

**Supplementary data**

Supplementary data are available at PCP online.

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