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REVIEW

SPECIAL ISSUE: PLANT CELL BIOLOGY

Actin-mediated movement of chloroplasts

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ABSTRACT

Plants are sessile and require diverse strategies to adapt to fluctuations in the surrounding light conditions. Consequently, the photorelocation movement of chloroplasts is essential to prevent damages that are induced by intense light (avoidance response) and to ensure efficient photosynthetic activities under weak light conditions (accumulation response). The mechanisms that underlie chloroplast movements have been revealed through analysis of the behavior of individual chloroplasts and it has been found that these organelles can move in any direction without turning. This implies that any part of the chloroplast periphery can function as the leading or trailing edge during movement. This ability is mediated by a special structure, which consists of short actin filaments that are polymerized at the leading edge of moving chloroplasts and are specifically localized in the space between the chloroplast and the plasma membrane, and is called chloroplast-actin. In addition, several of the genes that encode proteins that are involved in chloroplast-actin polymerization or maintenance have been identified. In this Review, we discuss the mechanisms that regulate chloroplast movements through polymerization of the chloroplast-actin and propose a model for actin-driven chloroplast photorelocation movement.

KEY WORDS: Actin filament, Actin polymerization, Blue light, Chloroplast, Chloroplast movement, Myosin, Phototropin

Introduction

The photorelocation movement of chloroplasts is one of the strategies that evolved to enable land plants to adapt to changing environmental light conditions. As plants are sessile organisms, they are unable to move away from strong sunlight or weak light conditions, for example under canopies. Light is absorbed by a blue light receptor, phototropin (phot), of which two are found in *Arabidopsis thaliana* (phot1 and phot2). Although some plants, such as those of the Oxalidaceae and Leguminosae families, can minimize the effects of direct sunlight through leaf nastic movements (Powles and Björkman, 1981; Rosa and Forseth, 1996), these are exceptional cases. In addition, climbing plants rapidly grow above canopy levels by means of vines, which allows their leaves to perceive light efficiently (Darwin, 1865; Ichihashi and Tateno, 2011). However, this strategy involving vines also only applies to specific types of plants. Therefore, plants must adapt to the environmental conditions of the places where they germinate and grow. Even for plants that have adapted to growth in open spaces, direct sunlight is too intense (Kasahara et al., 2002; Higa and Wada, 2016), which necessitates various measures to avoid light-induced damages or an efficient recovery from the damages that are caused by strong light (Niyogi, 1999). An essential way to ensure the protection of chloroplasts from photodamage is an avoidance movement of chloroplasts whereby they move from the cell surface (the periclinal wall) to a side wall (anticlinal wall) (Cazzaniga et al., 2013). Similarly, an accumulation movement of chloroplasts from areas with very weak light to those with relatively strong light is necessary for efficient photosynthetic activities (Wada, 2013, 2016) (Fig. 1). Chloroplasts also exhibit dark positioning, although here the positions of chloroplasts vary among different tissues (Wada, 2016). For example, chloroplasts move to the bottom of palisade cells (mesophyll cells underneath the epidermis) in seed plant leaves in response to darkness. In contrast, in young fern gametophytes (haploid stage plants that developed from germinated spores) of *Adiantum capillus-veneris*, which only have one cell layer, the chloroplasts move to the sidewalls (anticlinal walls) that connect neighboring cells, but are not localized at the bottom of cells (Kagawa and Wada, 1993, 1995) (Fig. 1). Strikingly, the function of dark positioning remains unknown, although early studies on the physiological significance of dark positioning proposed that it serves to supply nutrients to chloroplasts during the night (Senn, 1908).

Chloroplast movements were first observed in the 19th century, and a landmark study on these movements was performed early in the 20th century by Gustav Senn in Germany (Senn, 1908). Subsequently, chloroplast behavior in fern and moss gametophyte cells was analyzed by using a partial cell illumination system in combination with time-lapse imaging (Wada, 2016; Yatsuhashi et al., 1985). However, the most important advance in this field was achieved through the analysis of the mechanisms that underlie chloroplast movements in mutant *Arabidopsis* plants, which occurred only recently (Kong and Wada, 2011, 2014; Suetsugu and Wada, 2016).

Here, we describe the light-induced migration of individual chloroplasts, as well as the recently discovered actin structures that are specifically involved in chloroplast movements. Furthermore, we present a model of the force-generating mechanism that underlies these plant-specific, actin-based chloroplast movements.

Behaviors of an individual chloroplast

Chloroplast movement can be photometrically detected by analyzing red-light transmittance through a leaf (Wada and Kong, 2011). The protonemata (the filamentous structure comprising early haploid cells) and young prothalli (the heart-shaped gametophyte that produces both male and female gametes) of *A. capillus-veneris* are the prominent experimental systems used to study individual chloroplast behaviors in order to elucidate the mechanisms that are responsible for movement. Because fern gametophytes are not surrounded by any tissue, chloroplasts can be easily observed under a light microscope. Moreover, it is relatively easy to cultivate samples from spores on an agar plate (Wada and Furuya, 1970; Wada and Tsuboi, 2015).

In a vacuolated cell, the flat sides of planoconvex lens-shaped chloroplasts are able to attach to the plasma membrane and slide, but

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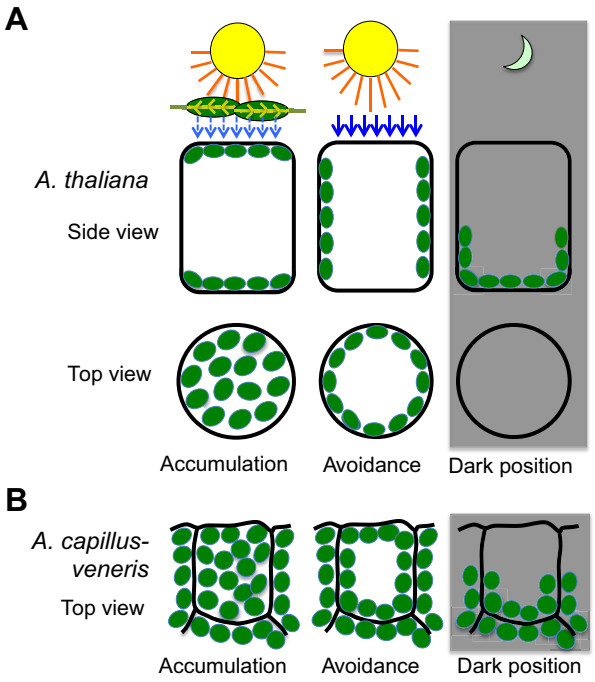

Fig. 1. Chloroplast movements in *Arabidopsis thaliana* and *Adiantum capillus-veneris* cells. (A) Left, weak light conditions under the canopy or owing to shade (accumulation response); middle, strong light conditions under direct sunlight (avoidance response); right, in darkness (dark positioning). Under weak light conditions, chloroplasts gather at the periclinal wall to absorb more light. When the light is strong, chloroplasts localize at the anticlinal walls to minimize photodamage. In darkness, chloroplasts of *A. thaliana* migrate to the bottom of cells, although the physiological purpose of this movement is unknown. (B) The dark positioning in cells of single layer prothallial cells of *A. capillus-veneris* is similar to the chloroplast distribution of the avoidance response, except in the outermost cells of prothallia. No chloroplasts exist at the cell margin in the dark cell, but many chloroplasts are found in cells undergoing an avoidance response.

not roll, in any direction along the plasma membrane. This indicates that chloroplasts do not have an innate head and tail, at least with respect to movement (Tsuboi and Wada, 2011; Tsuboi et al., 2009). Our previous work revealed that when a part of a periclinal wall of a dark-adapted fern prothallial cell was illuminated with a short pulse of light, chloroplasts that were positioned far from the beam-irradiated area started moving toward the light beam within a few minutes (Kagawa and Wada, 1994). Additionally, the area of the chloroplast periphery that was nearest to the irradiated spot served as the leading edge (i.e. head) of the moving chloroplast, which implies that any part of the chloroplast periphery can function as the front side (Tsuboi and Wada, 2011; Tsuboi et al., 2009). If a second beam of light was directed onto another part of the cell, the moving chloroplast changed directions and migrated toward the new irradiated spot without turning (Tsuboi et al., 2009). This indicates that the leading edge of the chloroplast changed to the area of its periphery that was closest to the second beam. Chloroplasts exhibit a similar behavior during an avoidance response that is induced by strong light, or dark positioning, which results from exposure to darkness (Tsuboi and Wada, 2011). Therefore, chloroplasts can move in any direction from the periclinal wall, and any part of the chloroplast periphery can serve as the leading edge. These results suggest that chloroplasts do not move along pre-existing tracks, although the cytoskeleton is nevertheless thought to contribute to the mechanisms that regulate movement.

Actin filaments for chloroplast anchoring

Actin filaments have been proposed to contribute to the ability of chloroplasts to move (Haupt, 2001; Takagi, 2003; Wada et al., 1993), and ring-like, basket-like or honeycomb actin filament-like structures and actin filaments that are associated with chloroplasts have been detected in ferns, mosses and seed plants (Dong et al., 1996; Kadota and Wada, 1989; Kandasamy and Meagher, 1999; Sato et al., 2001). For example, in protonemata of the moss *Physcomitrella patens*, the involvement of microtubules, as well as actin filaments, in red and blue light-induced chloroplast movements has been reported (Sato et al., 2001). Actin filaments have also been also visualized in fixed protonemata cells of *A. capillus-veneris* stained with Rhodamine-labeled phalloidin (Kadota and Wada, 1989, 1992). Here, a ring-like structure of ∼4 µm diameter appeared surrounding the chloroplast periphery at the plasma membrane side when the chloroplast accumulation response was induced by polarized red or blue light (Kadota and Wada, 1989), or by partial cell irradiation with a microbeam (Kadota and Wada, 1992). Likewise, when a part of a protonemal cell was irradiated with a strong microbeam, ring structures were observed around chloroplasts that accumulated on both sides of the microbeam (Kadota and Wada, 1992). The ring structure disappeared when the light was removed. Subsequently, a time-lapse study on the dynamics of the ring structure revealed that it appeared following the completion of the accumulation response and disappeared before the chloroplasts started to disperse in response to darkness (Kadota and Wada, 1992). Therefore, the ring structure is likely not involved in chloroplast movements, but instead influences the anchoring of chloroplasts to the plasma membrane (Kadota and Wada, 1992; Takagi et al., 2009). Consequently, if these actin structures do not affect chloroplast movements, the cytoskeletal structures that are involved in this process still need to be identified.

Actin filaments for chloroplast movement

Actin filaments and microtubules are long filaments, and their polarities enable them to transport organelles over long distances with the help of motor proteins, such as myosins and kinesins (Brandizzi and Wasteneys, 2013; Ueda et al., 2015). Around chloroplasts, even if such a fine organized meshwork of actin filaments or microtubules was present, transporting chloroplasts in any direction could prove difficult because filaments could hinder movement owing to the limited cellular space in combination with their polarities, which would limit the directionality of chloroplast movements. It is thus conceivable that a new cytoskeletal network is created around the microbeam-irradiated area just after chloroplast movement is induced. In this model, the newly formed cytoskeletal network would be expected to signal or spread from the irradiated area to all chloroplasts in a cell, thereby allowing the chloroplasts to move towards the beam of light. However, such a cytoskeletal structure has not yet been detected around a microbeam-irradiated area, even after thorough examination.

Our group have examined dynamic actin behaviors in live, rather than fixed cells, from transgenic *Arabidopsis* plants that expressed the green fluorescent protein (GFP) fused to the cytoskeletal protein TALIN that binds to filamentous actin (F-actin) (Box 1). In these experiments, we detected a structure that consists of dense short actin filaments at the leading edge of chloroplasts that exhibit an avoidance response (Kadota et al., 2009) (Fig. 2A,C). These chloroplast-actin (cp-actin) filaments surrounded chloroplasts, even those that were stationary and stable. Additionally, the abundance and distribution of the cp-actin filaments was affected by blue light

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Box 1. Caveats of cp-actin imaging in live plant cells

Cp-actin filaments are highly light-sensitive and thus are depolymerized quickly when exposed to lasers, such as the 488-nm laser that is used to observe fluorophores (e.g. GFP fused to actin-binding probes). Furthermore, cp-actin filaments are rapidly and reversibly reorganized within a minute (Kong et al., 2013a). Therefore, it has proven difficult to maintain the structure of cp-actin filaments during chemical fixation in multi-layered cells, such as *A. thaliana* palisade cells, as compared to in linear protonemata cells and the single-layered prothalli of the fern *A. capillus-veneris* (Tsuboi and Wada, 2012).

Actin probes such as TALIN (Kost et al., 1998), LIFEACT (a 17-amino-acid peptide from yeast Abp140; Riedl et al., 2008) and fABD2 (Sheahan et al., 2004), which are based on the second actin-binding domain of the actin bundler FIMBRIN, are widely used to visualize actin filaments in live cells. However, there are caveats with using each of these probes: TALIN expression might lead to the creation of actin artifacts within the cytoplasm (Ketelaar et al., 2004). LIFEACT has a high affinity for globular actin, thereby creating a high background fluorescence. In addition, it is not capable of highlighting numerous specialized subcellular structures that are comprised of actin, as the binding site for LIFEACT on actin might be masked in these (Munsie et al., 2009; Riedl et al., 2008; Sanders et al., 2013). Although the very prominent actin network is clearly probed with fABD2 in most cell types in *Arabidopsis* (Voigt et al., 2005), the use of fABD2 is also limited in its use for certain actin structures, such as cp-actin filaments which are only weakly labeled with fABD2 (Kong et al., 2013a). Furthermore, it is known that the expression levels of these probes are critical, and when expressed at even slightly elevated levels compared to wild-type, actin probes can lead to detrimental actin dynamics, as well as introduce cellular phenotypes (Voigt et al., 2005). In our experiments, transgenic lines that express these actin probes show normal chloroplast movement. However, it is nevertheless of some concern that the chloroplast-associated actin filaments are preferentially visible with TALIN, but only faintly with either LIFEACT or fABD2 (Kong et al., 2013a). Such a difference might come about because of the distinct binding affinities of each actin probe to cp-actin filaments, and a difference of the availability of binding sites in these filaments compared to those in cytoplasmic actin filaments. Indeed, cp-actin filaments have many specific features regarding their dynamics, further binding factors that have yet to be characterized and an influence on the light-induced signaling pathways in comparison to cortical and cytoplasmic actin filaments.

(Kadota et al., 2009; Kong et al., 2013a). As soon as part of a chloroplast was irradiated with strong blue light to induce an avoidance response, cp-actin filaments disappeared at the irradiated site, but were newly polymerized at the opposite side of the moving chloroplast (Kadota et al., 2009; Kong et al., 2013a) (Figs 2C and 3A). In contrast, during an accumulation response, cp-actin filaments were not depolymerized on the trailing side, and were detected over the entire chloroplast periphery, although there were small differences in their abundance between the leading and trailing edges (Kadota et al., 2009; Kong et al., 2013a) (Fig. 3B).

The cp-actin filaments in *A. capillus-veneris* prothallial cells have also been investigated (Tsuboi and Wada, 2012). The center of a dark-adapted prothallial cell was illuminated to induce a chloroplast accumulation response, and the behavior of the chloroplasts in the surrounding areas that migrated toward the irradiated area was analyzed by time-lapse imaging (Tsuboi and Wada, 2012). When the accumulation response was clearly observable, the prothallium was fixed and actin filaments were stained with phalloidin. The cp-actin filaments at the leading edge of moving chloroplasts were then examined (Tsuboi and Wada, 2012). Interestingly, it was found that the functions of actin filaments in the linear thread-like protonemal cells (Kadota and Wada, 1989, 1992) and those in prothallial cells differ (Tsuboi and Wada, 2012), with the former being required for

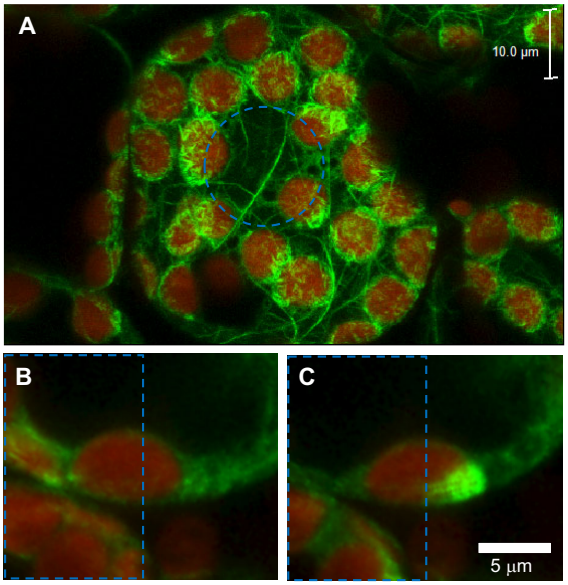

Fig. 2. Cp-actin filaments during the avoidance response in *Arabidopsis thaliana*. Part of a mesophyll cell was irradiated with a microbeam of strong blue light in the region indicated by the blue circle (A) or rectangle (B,C) with a dotted line. (A) This leads to the asymmetric distribution of cp-actin filaments and the migration of chloroplasts away from the irradiated area. (B,C) The side view of cp-actin filaments clearly shows that they are present between the chloroplast and the plasma membrane at the leading edges of moving chloroplasts. Cp-actin filaments are not found before the irradiation by blue light (B), but clearly seen after irradiation for 5 min (C). The fluorescent images are pseudo-colored to indicate actin filaments (green) and chloroplast (red). Scale bars: 10 µm (A), 5 µm (B,C).

anchoring chloroplasts and the latter being involved in chloroplast movements. The variability in the structures and predicted functions of the actin filaments in both cases appear to represent the underlying differences in these cell types. We believe that these structures are not artifacts that are induced by chemical treatments, because the protocols are almost identical (Kadota and Wada, 1989, 1992; Tsuboi and Wada, 2012). However, live-imaging of actin is needed to reveal the actual cytoskeletal changes that are related to chloroplast movements in *A. capillus-veneris*. Unfortunately, a reliable method for generating stable transgenic fern gametophytes has not yet been developed.

Cp-actin filaments are not easily detected because of several factors. First, their asymmetric distribution is clearly visible only when chloroplasts are moving, especially during the avoidance response (Kadota et al., 2009; Kong et al., 2013a) (Fig. 2). When a chloroplast migrates away from a strong microbeam-irradiated area and stops moving, the cp-actin structure is reorganized over the entire chloroplast periphery because of the cp-actin polymerization that appears to happen at the trailing edge (Kadota et al., 2009; Kong et al., 2013a). Second, cp-actin filaments are easily destroyed by chemical fixation, at least those of *A. thaliana*. However, the cryofixation and freeze-substitution treatments successfully applied to leaf cells (Kandasamy and Meagher, 1999) may preserve cp-actin filaments. Third, although we are able to visualize cp-actin filaments with GFP–TALIN, the widely used actin probes LIFEACT and fABD2 are less suitable for visualizing cp-actin filaments (Kong et al., 2013a; Whippo et al., 2011) (see Box 1). The differences between actin probes might be caused by them having distinct cp-actin-binding activities. Furthermore, the difficulties in recognizing cp-actin might also be due to technical parameters associated with microscopy (Kong et al., 2013a) (see Box 1). Cp-

actin filaments are very fragile and are easily degraded. Thus, when cp-actin filaments are irradiated with strong light during imaging, they might be depolymerized very quickly. The dynamics of cp-actin filaments polymerization and their maintenance thus remain to be investigated. In the following sections, we discuss several factors that have been reported to contribute to cp-actin polymerization and/or maintenance.

Factors for the polymerization and/or maintenance of cp-actin filaments

Because cp-actin filaments are thought to be essential for chloroplast movements, mutant lines that exhibit defective chloroplast movements have been extensively examined. These analyses have considerably advanced our understanding of the regulatory roles of several protein components in cp-actin polymerization and/or maintenance, and its force-generating mechanism (Kong and Wada, 2011, 2014; Suetsugu and Wada, 2016).

A central function for CHLOROPLAST UNUSUAL POSITIONING 1 (CHUP1) was proposed following analysis of *A. thaliana chup1* mutants, in which chloroplasts were detached from the plasma membrane and were deposited at the bottom of cells and did not move under any light condition (Oikawa et al., 2003). The CHUP1 protein consists of 1004 amino acids with various functional domains, including a hydrophobic region for binding to the chloroplast outer membrane, a coiled-coil region for anchoring the chloroplast to the plasma membrane, an F-actin-binding site, and a C-terminal conserved region (Oikawa et al., 2003, 2008). Importantly, CHUP1 binds to profilin (Schmidt von Braun and Schleiff, 2008), which is a small actin-binding protein that supports actin assembly at the barbed end. Consequently, *chup1* mutant cells lack cp-actin filaments (Kong et al., 2013a). In addition, CHUP1 only functions in actin-mediated movement, but not in microtubule-mediated chloroplast movement in *P. patens* (Usami et al., 2012). This suggests that CHUP1 is involved in the polymerization of cp-actin for chloroplast movement (Oikawa et al., 2003; Schmidt von Braun and Schleiff, 2008; Wada and Suetsugu, 2004).

Similarly, KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT (KAC) is necessary for cp-actin-dependent chloroplast movements, although its exact molecular mechanism in this process remains unknown (Suetsugu et al., 2010b). The KAC motor domain is similar to that of kinesins; however, it binds to F-actin under *in vitro* conditions and lacks a microtubule-binding activity. Just like CHUP1, KAC is involved in actin-mediated, but not in microtubule-mediated chloroplast movement in *P. patens* (Shen et al., 2015; Suetsugu et al., 2012). The *A. thaliana* genome includes two *KAC* genes, *KAC1* and *KAC2*. Our previous work showed that *kac1 kac2* double mutant plants do not contain any cp-actin filaments, and the chloroplasts are detached from the plasma membrane (Suetsugu et al., 2010b). Interestingly, the chloroplasts in *kac1 kac2* double mutant leaves exhibited a strong avoidance response even in the absence of cp-actin filaments, whereas we did not detect an accumulation response (Suetsugu et al., 2016). The mechanism regulating the avoidance response of chloroplast movements in *kac1 kac2* double mutant plants has not been further characterized. Therefore, whereas cp-actin filaments are required for fast, directional chloroplast movements, another actin-dependent mechanism controls the blue-light induced avoidance response, at least in the case of loss of both KACs (Suetsugu et al., 2016). A possible compensation pathway through cytoplasmic actin filaments is conceivable, as nuclei in pavement cells employ cytoplasmic actin for their avoidance response (Higa et al., 2014). However, this awaits confirmation in chloroplasts.

The functions of PLASTID MOVEMENT IMPAIRED1 (PMI1), which is a plant-specific C2-domain protein (DeBlasio et al., 2005), and two PLASTID MOVEMENT IMPAIRED1-RELATED (PMIR) proteins (PMIR1 and PMIR2) (Suetsugu et al., 2015) might differ from those of CHUP1 and KAC. Our studies have revealed that, in *pmi1* mutant cells that are exposed to strong light, chloroplasts do not move away from the light and there are no observable cp-actin filaments. However, after a 4-min incubation in darkness, cp-actin filaments appeared, but then disappeared again during a subsequent treatment with strong light. These changes were consistent with those seen in wild-type plants, but occurred more quickly (Suetsugu et al., 2015). The appearance and disappearance of cp-actin filaments under dark and strong light conditions, respectively, were repeatedly observed. This indicates that PMI1 might be involved in the maintenance, rather than the initial polymerization of cp-actin. Interestingly, whereas it is unclear whether the PMI1 homologs PMIR1 and PMIR2 influence chloroplast movements, these two proteins are necessary for nuclear movement in pavement cells (Suetsugu et al., 2015).

THRUMIN1 is a light-regulated actin-bundling factor that is involved in chloroplast movement. The protein contains an intrinsically disordered region in the N-terminal half, and a glutaredoxin-like and putative zinc-binding cysteine-rich domain in the C-terminal half. THRUMIN1 localizes to the plasma membrane through the myristoylated N-terminus, and decorates actin filaments in a blue light- and phototropin-dependent manner (Whippo et al., 2011). Importantly, THRUMIN1 also colocalizes with cp-actin filaments during the avoidance response. Moreover, *thrumin1* mutant cells exhibit defective reorganization of cp-actin filaments during the avoidance response (Kong et al., 2013a). Thus, THRUMIN1 is important for the functionality of cp-actin filaments, although the relevant mechanism has not yet been elucidated.

There are additional factors that have been shown to affect cp-actin filament dynamics, and these include J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE1 (JAC1) (Ichikawa et al., 2011; Suetsugu et al., 2005) and the WEAK CHLOROPLAST MOVEMENT UNDER BLUE LIGHT1–PLASTID MOVEMENT IMPAIRED 2 (WEB1–PMI2) complex (Kodama et al., 2010; Luesse et al., 2006). Although each aforementioned protein has not been fully functionally characterized, mutants that are deficient in JAC1 and WEB1–PMI2 exhibit partial defects in chloroplast movement (Kodama et al., 2010; Luesse et al., 2006; Suetsugu et al., 2005). Consistent with this, dynamic reorganizations of cp-actin filaments are partially defective in the cells mutant for JAC1 and WEB1–PMI2 (Ichikawa et al., 2011; Kodama et al., 2010). Therefore, these protein factors function in the modulation of blue light-induced cp-actin dynamics, rather than being directly involved in cp-actin polymerization and/or depolymerization.

Regulation of directional chloroplast movement

Despite the identification of several components that are involved in the polymerization or maintenance of cp-actin, it is still not clear how the direction of movement is controlled. As mentioned above, chloroplasts do not have an innate head and tail, and can move in any direction (Fig. 3A,B). Dumbbell-shaped chloroplasts are common, especially in fern gametophytes, and are also able to move in any direction (Tsuboi and Wada, 2012). Additionally, chloroplasts in the prothalli of *A. capillus-veneris*, which migrate toward a microbeam for an accumulation response or away from strong light for an avoidance response, move directly towards the destination without turning. While moving, chloroplasts are

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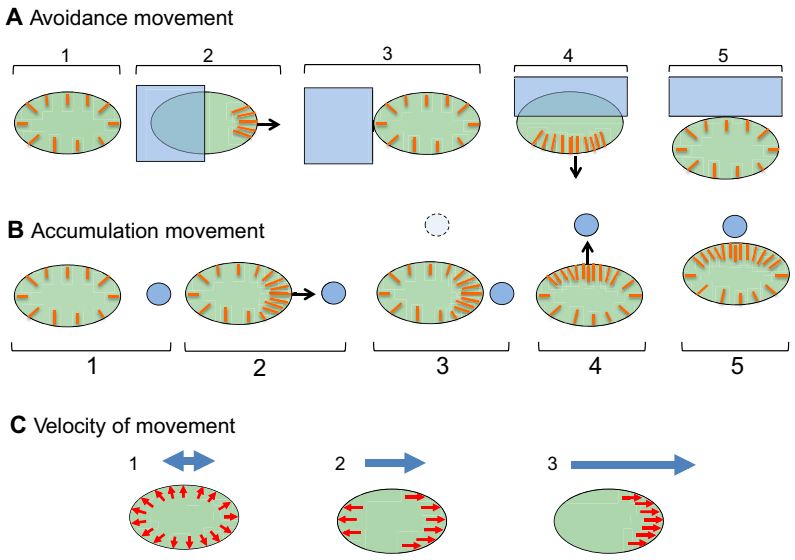

Fig. 3. Cp-actin filaments in the directional and velocity control of chloroplast movements. (A) Avoidance movement induced by strong light. When half of a stable chloroplast (1) is illuminated with strong blue light [blue rectangle, (2)], cp-actin filaments (orange bars) at the irradiated part disappear and new cp-actin filaments polymerize at the opposite side (i.e. leading edge) (2). After the chloroplast has moved away from the irradiated area, cp-actin filaments become evenly distributed (3). If a second beam of light is applied to a different half of the chloroplast (see diagram), the asymmetric distribution of cp-actin filaments is re-established and the avoidance response re-occurs (4,5). (B) Accumulation movement induced by weak light. When part of a periclinal wall is illuminated with a microbeam (1) of either high or low intensity blue light (or red light in the case of ferns), chloroplasts that are positioned far from the spot migrate toward the irradiated area with an increased abundance of cp-actin filaments at the leading edge (2,3). The cp-actin filaments at the trailing edge are not depolymerized. If a second microbeam is applied (3, dotted circle), the moving chloroplast changes directions, with an increased abundance of cp-actin filaments at the new leading edge (4,5). (C) Velocity control through the balance of cp-actin filament amounts at the leading and trailing edges of a chloroplast. Red arrows indicate the vectors of centrifugal forces caused by cp-actin filament polymerization around the chloroplast periphery. (1) Stable chloroplast, for which the cp-actin filaments are evenly distributed around the chloroplast periphery so that all vector signals cancel each other. (2,3) In chloroplasts moving toward the right, their velocities are based on the sum of the centrifugal forces from the center to the chloroplast periphery. Blue arrows indicate the forces driving chloroplast movement.

continuously monitoring the light signal that is released by photoreceptors (Tsuboi and Wada, 2013). For example, chloroplasts move towards an area that is irradiated with a short pulse of red microbeam light (Tsuboi and Wada, 2013). This light is absorbed by neochrome, a chimera photoreceptor that consists of a phytochrome chromophore-binding domain in its N-teminus and phototropin in its C-terminus, so that this chimera is activated by red light and inactivated by far-red light. When the moving chloroplasts are illuminated by a far-red light pulse, they stop moving, but they restarted movement towards an area that was irradiated with a second red microbeam (Tsuboi and Wada, 2013). This result indicates that chloroplasts move as long as they are continually monitoring the signal from the photoreceptors.

When *A. thaliana* chloroplasts change their leading edges during movement, the distribution of cp-actin filaments gradually changes from the initial to the new leading edge of the chloroplast (Kong et al., 2013a). However, it is unlikely that the cp-actin filaments actually move from the initial leading edge to the new one. Instead, our careful observations suggest that *de novo* cp-actin filaments are polymerized at the emerging leading edge (Kong et al., 2013a), which indicates that an actin nucleation factor must be activated by the signal that is released by photoreceptors in order to initiate cp-actin polymerization. In other words, a receptor for this signal transduction that emanates from the photoreceptors must be localized around the chloroplast periphery to relay the signal to such an actin nucleation factor (Fig. 4).

Velocity of chloroplast movement

In addition to the direction of movement, its velocity is also precisely regulated by light intensity and photoreceptor numbers (see below). Chloroplasts move very slowly, at ∼1 µm per min, during both accumulation and avoidance responses (Kagawa and Wada, 1996; Tsuboi and Wada, 2011; Tsuboi et al., 2009). In these studies, chloroplast movements were induced by irradiating prothallial cells of *A. capillus-veneris* with a blue or red microbeam, or treating palisade cells of *A. thaliana* with a blue microbeam. Chloroplast movements were recorded every 15 s with time-lapse imaging under infrared light, after which the velocity of movement was calculated. The velocity of chloroplast movements during the accumulation response was independent of the fluence rate (light intensity), as it was approximately the same under various tested microbeam light intensities (Kagawa and Wada, 1996; Tsuboi et al., 2009; Tsuboi and Wada, 2011). In contrast, the velocity of chloroplast movements during an avoidance response in *A. thaliana* cells treated with blue light was proportional to the light fluence rate (Kagawa and Wada, 2004). Additionally, chloroplasts in a heterozygous *phot2* mutant line moved ∼50% slower than in wild-type cells, which suggests that the velocity is dependent on cellular phot2 levels (Kagawa and Wada, 2004). The velocity of chloroplast movements during an avoidance response was observed to be slightly lower in *A. capillus-veneris* prothallial cells than in the palisade cells of *A. thaliana* cells, but still exhibited similar characteristics (Tsuboi and Wada, 2011). When chloroplasts in transgenic *A. thaliana* plants that are expressing GFP–TALIN were irradiated with a microbeam of strong blue light, the cp-actin filaments disappeared quickly at the irradiated sites, but were extensively polymerized at the non-irradiated chloroplast parts. These findings are consistent with an asymmetric distribution of cp-actin filaments and the establishment of a leading edge of moving chloroplasts (Fig. 2A,C; Fig. 3A–C). The difference in the

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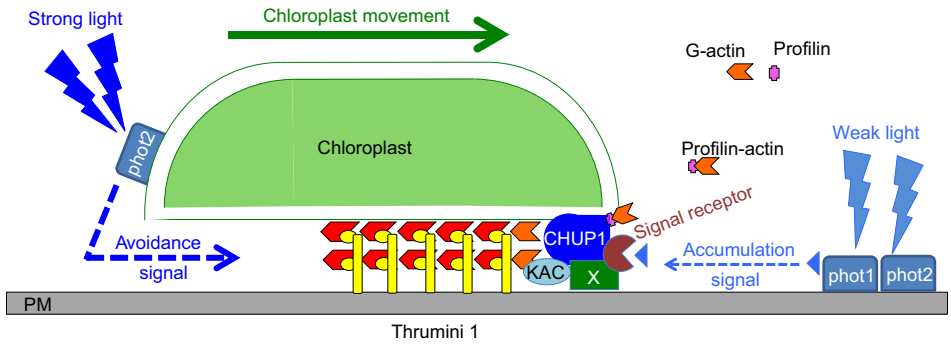

Fig. 4. Model of the molecular mechanisms that underlie cp-actin-based chloroplast movements. The phot2 protein, which functions as a photoreceptor for the avoidance response, localizes to the outer envelope of the chloroplast. Phot1 and phot2, which mediate the accumulation response, localize to the plasma membrane. Signals for the accumulation and avoidance response are released from the photoreceptors and are received by a signal receptor that is probably associated with CHUP1, which anchors the chloroplast to the plasma membrane through an unknown membrane protein (labeled X). A potential CHUP1-dependent polymerization of cp-actin filaments is initiated by the incorporation of profilin-bound actin. KAC acts to promote cp-actin polymerization together with CHUP1 or in the maintenance of cp-actin filaments, at least in the accumulation response. Polymerized cp-actin filaments are bundled by THRUMIN1, which localizes to the plasma membrane, so that cp-actin filaments are fixed to the plasma membrane. Thus, the chloroplast slides forward by newly polymerized cp-actin as long as cp-actin is polymerized. Other factors (e.g. PMI1 and WEB1) that are believed to regulate cp-actin dynamics are not included in this model because their exact molecular functions and the relationship with other factors are unclear.

abundance of cp-actin filaments between the leading and trailing edges was proportional to the velocity of chloroplast movements during the avoidance response (Kadota et al., 2009; Kong et al., 2013a). Thus, the velocity of the avoidance movement by *A. thaliana* chloroplasts depends on amounts of activated phot2 and, consequently, the difference in the amount of cp-actin filament at the leading and trailing edges (Fig. 3C). It remains to be determined whether this is also true in other plants.

Towards a mechanism for cp-actin filament-dependent movements

Although the exact nature of the cascades that trigger chloroplast movement is not well understood, it is clear that the accumulation and avoidance responses are dependent on different, or at least partially independent, signal transduction pathways. The signal for the accumulation response can be transmitted over longer distances and remains active longer than the avoidance response signal (Higa and Wada, 2015; Kagawa and Wada, 1999). Microbeam irradiation and biochemical studies suggest that these differences in accumulation and avoidance responses are associated with the intracellular localization of the blue light receptors. Specifically, phot1 and phot2, which are required for accumulation response, are located on the plasma membrane (Kagawa and Wada, 1994), and phot2, which is involved in avoidance response, is found on the chloroplast envelope (Kong et al., 2013b; Tsuboi and Wada, 2011). Thus, increased light intensity directly activates more phot2 on the chloroplast, whereas in the accumulation response, the light signal that is perceived through phot1 and phot2 on the plasma membrane might be relayed differently towards the chloroplast. However, we believe that the force-generating mechanisms for both accumulation and avoidance responses are in fact the same, because proteins (e.g. CHUP1, PMI1 and THRUMIN1) that mediate cp-actin polymerization and/or maintenance are indispensable during the accumulation and avoidance responses.

Force generation and movement of cargo and organelles are commonly mediated by cytoskeletal motor protein, in particular myosins (Buchnik et al., 2015). Importantly, double-, triple- and quadruple-knockouts of four major myosin XI genes that are expressed in leaf cells inhibit the movement of all organelles except for chloroplasts (Suetsugu et al., 2010a). Although these data argue for a myosin-independent process, this has to await a full characterization of a mutant plant that lacks all myosin paralogs. Nevertheless, the polymerization of cp-actin filaments represents the most likely mechanism to generate force for chloroplast movement. Chloroplasts are anchored to the plasma membrane by the N-terminal coiled-coil domain of CHUP1 (Fig. 4) (Oikawa et al., 2008). Concurrently, the dynamic reorganization of cp-actin filaments is regulated by CHUP1 and other factors, such as KACs, WEB1–PMI2, PMI1 and PMIR, and THRUMIN1 (Kadota et al., 2009; Kodama et al., 2010; Kong et al., 2013a; Suetsugu et al., 2015, 2010b). Polymerized cp-actin filaments might subsequently be bundled by THRUMIN1 and simultaneously anchored to the plasma membrane (Whippo et al., 2011). Then, the profilin–actin complex is captured and G-actin is incorporated for cp-actin filament polymerization by CHUP1 and other factors that could play a role as the cp-actin nucleator(s) (Fig. 4). The cp-actin filaments are bundled through plasma membrane-anchored THRUMIN1. The cp-actin filaments may thereby be bound firmly to the plasma membrane by THRUMIN1, so that CHUP1 on the chloroplast, along with other factors, will slide the chloroplast forward through force creation by polymerizing numerous new cp-actin filaments at the edge of the chloroplast (Fig. 4). Such a polymerization-driven force has been calculated to be at ∼1–9 piconewtons (Dmitrieff and Nédélec, 2016), which appears to be high enough to enable these movements.

Conclusions and future perspectives

The discovery of cp-actin filaments that specifically regulate chloroplast movements has opened new possibilities for research into the migration of plant organelles. A recent study concluded that nuclear movements in pavement cells are also mediated by cp-actin filaments that are polymerized on plastids (Higa et al., 2014). The most important issue regarding the mechanism that underlies actin-dependent chloroplast movements is how the motive force for chloroplast movements is generated without myosins. The proposed model of the force-generating mechanism (Fig. 4) is similar to the Arp2/3-dependent mechanism that mediates bacterial movement in animal cells with regards to the force that the polymerization of actin creates for the translocation of an object (Goley and Welch, 2006). However, the obvious difference between the two systems is that the

Arp2/3 complex exists at the rear part of bacteria (i.e. a 'comet tail'), whereas the cp-actin filaments are located at the leading edge of chloroplasts. Arp2/3-deficient mutants exhibit normal chloroplast movements (Kadota et al., 2009), indicating that Arp2/3 is not associated with cp-actin polymerization. Consequently, a mechanism that is yet to be discovered might regulate the movement of organelles. Fortunately, several factors have already been shown to be involved in cp-actin polymerization and/or maintenance, and CHUP1 represents the most important candidate for future investigations aimed at unveiling this mechanism. Furthermore, the ongoing work on the functions of other relevant factors may eventually necessitate a revision of the current model for chloroplast movements.

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Competing interests
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