Two kinesin-like proteins mediate actin-based chloroplast movement in *Arabidopsis thaliana*

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Organelle movement is essential for efficient cellular function in eukaryotes. Chloroplast photorelocation movement is important for plant survival as well as for efficient photosynthesis. Chloroplast movement generally is actin dependent and mediated by blue light receptor phototropins. In *Arabidopsis thaliana*, phototropins mediate chloroplast movement by regulating short actin filaments on chloroplasts (cp-actin filaments), and the chloroplast outer envelope protein CHUP1 is necessary for cp-actin filament accumulation. However, other factors involved in cp-actin filament regulation during chloroplast movement remain to be determined. Here, we report that two kinesin-like proteins, KAC1 and KAC2, are essential for chloroplasts to move and anchor to the plasma membrane. A *kac1* mutant showed severely impaired chloroplast accumulation and slow avoidance movement. A *kac1kac2* double mutant completely lacked chloroplast photorelocation movement and showed detachment of chloroplasts from the plasma membrane. KAC motor domains are similar to those of the kinesin-14 subfamily (such as Ncd and Kar3) but do not have detectable microtubule-binding activity. The C-terminal domain of KAC1 could interact with F-actin in vitro. Instead of regulating microtubules, KAC proteins mediate chloroplast movement via cp-actin filaments. We conclude that plants have evolved a unique mechanism to regulate actin-based organelle movement using kinesin-like proteins.

cp-actin | blue light | organelle movement | phototropin

Organelle movement is pivotal for efficient cellular functions in plants as well as in animals and fungi (1). Chloroplast photorelocation movement is essential for the adaptation of plants under the fluctuating natural light conditions (2). Chloroplasts accumulate toward light under low-light conditions (accumulation response) to maximize light capture for photosynthesis, and they escape from intense light (avoidance response) to avoid chloroplast photodamage (2, 3). These responses are mediated by blue light receptor phototropins in green algae, mosses, and ferns as well as in seed plants (2). *Arabidopsis thaliana* expresses two phototropins (4–6); phot1 and phot2 are required for the accumulation response whereas phot2 alone is essential for the avoidance response.

Like other plant organelle movements, chloroplast photorelocation movement generally is dependent on actin filaments rather than on microtubules (2). Our recent study suggested that chloroplast photomovement and anchorage to the plasma membranes are mediated by short actin filaments on chloroplasts (cp-actin filaments); formation of cp-actin filaments is associated with phototropin-mediated chloroplast movement (7). The chloroplast outer membrane protein, chloroplast unusual positioning 1 (CHUP1), which can bind to both F-actin and G-actin in vitro (8–10), is implicated in cp-actin filament formation (7). The correlation of cp-actin signal with the velocity of chloroplast movement suggests that cp-actin filaments play a role in generating the motive force (7), but it remains to be determined if cp-actin filaments actually provide the motive force. To identify other components involved in cp-actin filament regulation during chloroplast movement, we screened mutants deficient in chloroplast photorelocation movement using a previously developed band assay (4, 8, 11). Here, we characterized a kinesin-like protein for actin-based chloroplast movement 1 (*kac1*) mutant.

Results

Positional Cloning of *KAC1* Gene and Characterization of *kac* Mutants. Two independent *kac1* alleles, *kac1-1* and *kac1-2*, were isolated (Fig. 1*A*). A map-based approach revealed that the *KAC1* gene is *At5g10470* (Fig. S1 *A* and *B*). Analyses of a transfer DNA (T-DNA) insertion line of *KAC1* (*kac1-3*) (Figs. S1*B* and S2 *A* and *B*) and genetic complementation of *kac1-1* with an *At5g10470* genomic construct confirmed that *KAC1* is *At5g10470*. Moreover, *Arabidopsis thaliana* has one gene that is highly similar to *KAC1*; this gene is named "*KAC2*" (*At5g65460*) and is located on the lower arm of chromosome 5 (Fig. S1*B*). Note that KAC1 was identified previously as a protein interacting with various proteins implicated in cell-cycle and microtubule function: CDKA1-interacting protein [designated "KLP2/KCA1" (12, 13)], the geminivirus protein AL1-interacting protein [designated "GRIMP" (14)], and the katanin-interacting protein [designated "KSN1" (15)]. In this paper we refer to KLP2/KCA1/GRIMP/KSN1 as "KAC1" and KCA2 as "KAC2." Although we cannot exclude completely the possibility that KAC proteins regulate cell-cycle or microtubule-dependent cell growth, under our growth conditions we could not detect obvious growth or developmental defects even in the *kac1kac2* double mutant. Moreover, observations of microtubule dynamics using GFP-TUA6 showed that no gross or obvious defect could be observed without a more quantitative analysis (Movie S1 and Movie S2). However, we cannot exclude the possibility that these KAC1 interactive proteins are involved in chloroplast movement.

To investigate the function of both KAC1 and KAC2 in chloroplast photorelocation movement, single and double mutants (Figs. S1*B* and S2*A*) were examined in detail. If not specified otherwise, *kac1-1* and *kac2-1* were used in all experiments as representative lines for *kac1* and *kac2*, respectively, because similar results were obtained using other *kac1* or *kac2* alleles. When dark-adapted wild-type and *kac* mutant plants were irradiated with a low-fluence (LL) or high-fluence (HL) white light for 2 h, typical chloroplast photorelocation movement was observed in mesophyll cells of both the wild type and *kac2*. Chloroplasts

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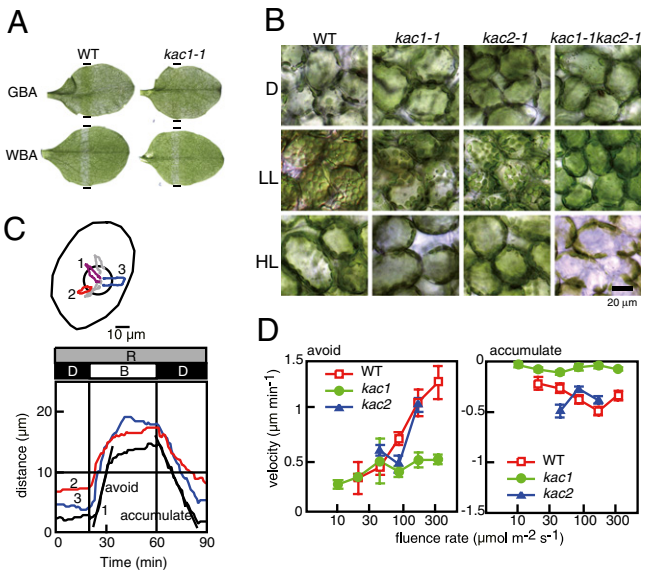

Fig. 1. *kac* mutants are deficient in chloroplast photorelocation movement. (*A*) Green (GBA) and white band assay (WBA) for detection of chloroplast accumulation and avoidance responses, respectively. The irradiated area is indicated on both sides of the leaves by black bars (*ca*. 1 mm). (*B*) Characterization of chloroplast photorelocation movement in mesophyll cells of *kac* mutants. Plants that were dark-adapted for about 12 h (D) were irradiated with white light at 10 (LL) or 100 μmole m^{-2} s^{-1} (HL) for 2 h. All micrographs were taken at the focal plane of the anticlinal wall closest to the epidermis. (*C* and *D*) Velocity of chloroplast photorelocation movement in mesophyll cells of wild-type, *kac1-1*, and *kac2-1*. (*C*) Chloroplast movement was induced by irradiation with a blue light microbeam (circle) at various fluence rates (11, 22, 49, 94, 188, and 376 μmole m^{-2} s^{-1}), and the movement of each chloroplast was traced during the experiments (*Upper*). Avoidance response was induced during microbeam irradiation, whereas accumulation response was induced after the microbeam turned off. The distances between the center of the microbeam and chloroplasts were plotted as a function of time (black, red, and blue lines), and the velocity was calculated as the slope (indicated by black lines) of the traveled distance during the avoidance or accumulation (*Lower*). (*D*) The velocity was plotted as a function of the fluence rate. Bar indicates SE. *P* values by two-tailed Student's *t* test were determined for fluence rates of 22, 49, 94, 188, and 376 μmole m^{-2} s^{-1}: for *kac1* versus wild type for avoidance response (*P* = 0.855, *P* = 0.706, *P* = 0.001, *P* = 0.001, *P* = 5.7E-05, respectively), for *kac1* versus wild type for accumulation response (*P* = 9.0E-05, *P* = 0.036, *P* = 7.8E-06, *P* = 5.8E-09, *P* = 2.0E-05, respectively), for *kac2* versus wild type for avoidance response (49, 94, and 188 μmole m^{-2} s^{-1}) (*P* = 0.062, *P* = 0.03, *P* = 0.885, respectively), and for *kac2* versus wild type for accumulation response (*P* = 0.007, *P* = 0.051, *P* = 0.058, respectively).

were positioned on the cell bottom in darkness (dark positioning) and moved to the cell surface under LL conditions in an accumulation response and were at the anticlinal wall under HL conditions in an avoidance response (11) (Fig. 1*B*). By contrast the dark positioning and avoidance response were normal in *kac1* mutants, but fewer chloroplasts accumulated at the cell surface under LL conditions (Fig. 1*B*), indicating that the *kac1* mutant is partially defective in the accumulation response (Fig. 1*B*). In the *kac1kac2* double mutant, the distribution of chloroplasts under both LL and HL conditions was similar to that of wild-type plants under HL conditions (Fig. 1*B*), indicating that *kac1kac2* is defective in the accumulation response. To analyze the avoidance movement in *kac1kac2* plants, a small part of the anticlinal wall of a mesophyll cell where chloroplasts localize in *kac1kac2* was irradiated with a microbeam of strong blue light, and chloroplast movement was recorded using a video camera. Wild-type chloroplasts escaped from the microbeam-irradiated area (Movie S3), but *kac1kac2* chloroplasts did not (Movie S4), indicating that *kac1kac2* is deficient in the avoidance response as well as the accumulation response.

***KAC1* and *KAC2* Genes Are Functionally Equivalent but Have Different Expression Levels.** When the velocity of chloroplast avoidance and accumulation were measured and plotted as the function of the fluence rate of the irradiated microbeam, as described previously (16) (see legend of Fig. 1*C* for details), the velocity in the wild-type chloroplasts increased proportionally to the fluence rate; the relationship was clear for the avoidance movement and slight for the accumulation movement (Fig. 1*D*). However, in the *kac1* mutant, the chloroplast accumulation response was hardly detected (Fig. 1*D* and Movie S5). Interestingly, the velocity of the avoidance movement was the same as that of the wild type in response to the lower fluence rates of the strong blue light (11, 22, and 49 μmol m^{-2} s^{-1}) but saturated at around 49 μmol m^{-2} s^{-1}. Hence, the velocity of avoidance in wild-type chloroplasts was twice or more than that in *kac1* at the higher fluence rates (188 and 376 μmol m^{-2} s^{-1}), indicating that *kac1* shows partial impairment of the avoidance response. The *kac2* mutant plants showed almost normal chloroplast photorelocation movement (Fig. 1*D*). RT-PCR analyses revealed that both *KAC1* and *KAC2* transcripts were expressed in various tissues (Fig. S2*C*). However, the microarray data showed that *KAC1* was more highly expressed than *KAC2*, especially in chloroplast-containing tissues. The KAC protein level was examined in wild-type, *kac1*, *kac2*, and *kac1kac2* using KAC antiserum (which can detect KAC1 and KAC2 equally in immunoblotting after SDS/PAGE) (Fig. S3 *A* and *B*). A band of approximately 150 kDa, corresponding to the size of full-length KAC proteins, was detected in wild-type and *kac2* mutant plants at a similar level, but a severely reduced level was observed in *kac1* plants, and the band was not detected in *kac1kac2* mutant plants, indicating that KAC1 proteins accumulated at a level several times higher than that of KAC2 (Figs. S3*B* and S4*A*). When *KAC2* cDNA was expressed under the control of the *KAC1* promoter and UTRs in *kac1kac2* mutant plants (*KAC1pro-KAC2*), KAC2 proteins accumulated at a level similar to that of KAC1 proteins in the *kac2* mutant and in the *KAC1* cDNA-expressing lines in the *kac1kac2* background (*KAC1pro-KAC1*) used as a control line (Fig. S4B). Chloroplast photorelocation movement and positioning were rescued to the same extent in both *KAC1pro-KAC1* and *KAC1pro-KAC2* lines but not in vector lines (Fig. S4 *C* and E), indicating that KAC1 and KAC2 are functionally equivalent.

KAC Proteins Were Detected in both Soluble and Membrane Fractions. Immunoblot analyses of fractionated proteins revealed that KAC proteins were detected in both soluble and microsomal fractions with a slightly larger amount detected in the soluble fraction, in contrast to phototropins and CHUP1, which were detected predominantly in the microsomal fraction (Figs. S3*C* and S4*A*). In transgenic lines expressing a *GFP-KAC1* or *KAC1-GFP* fusion gene under the control of the *KAC1* promoter and UTRs in the *kac1* background, normal chloroplast photorelocation movement was observed, and GFP-KAC1 or KAC1-GFP fluorescence was detected mainly in the cytosol and not in any particular organelles or cytoskeletons (Movie S6). Fractionation profiles and the abundances of KAC proteins were not different from those of the wild type or various mutants deficient in chloroplast movement (Fig. S3*C*). The fractionation profiles and abundances of phototropins and CHUP1 in *kac* mutants were also the same as those of the wild type (Fig. S4*A*), indicating that the defects of these mutants in chloroplast movement are not obviously attributed to impaired accumulation or localization of the assayed proteins regulating chloroplast movement.

***KAC* Genes Encode Kinesin-Like Proteins.** A homology search revealed that *KAC* genes encode kinesin-like proteins (Fig. 2,

Fig. S5). Among the 14 kinesin families (17, 18), KAC proteins belong to the kinesin-14 family. Some are known as minus end-directed motors bearing a motor core domain at their C terminus. The kinesin-14 family is subdivided into two large subfamilies, kinesin-14A and kinesin-14B, and kinesin-14B is further subgrouped into five groups (KIFC2/KIFC3, KatD, KCBP, KIF25, and unclassified) (17, 18). The 18 *A. thaliana* kinesin-14B members are divided into the KatD (10 members), KCBP (one member), and unclassified (seven members) subgroups. The KAC proteins belong to the unclassified kinesin-14B group (18, 19). The KAC protein is plant specific and exists from moss to seed plants (Fig. S6). Interestingly, KAC proteins have their motor core domain at the N terminus, although it is at the C terminus or middle region in most members of the kinesin-14 family. The KAC proteins are predicted to contain coiled-coil domains at the N-terminal region of the motor core domain and at the middle region of the proteins (Fig. 2). Although the KAC C-terminal domain does not show similarity to any other protein, this region is highly conserved among KAC proteins from various plant species (Fig. 2 and Fig. S6), suggesting that the KAC C-terminal domain is essential for the protein function.

Because KAC proteins are members of the kinesin-14 family, ATP-dependent binding to microtubules responsible for microtubule-based motor activity was examined using recombinant KAC motor core domains with or without the N-terminal coiled-coil domain. However, none of the recombinant KAC motor core domains cosedimented with microtubules under any of the nucleotide conditions (Fig. S7). Furthermore, we could not detect intrinsic ATPase activity of KAC proteins. Close inspection of the protein sequences corresponding to the KAC motor domains revealed changes of several amino acids necessary for motor activity and microtubule binding (*Discussion*).

KAC1 Neck/Core Interaction Mutations Affected KAC1 Protein Accumulation but Not the Directionality of Chloroplast Movement. Neck/motor core interactions are essential for the directionality of minus end-directed motor Ncd (20). Mutations of interacting amino acids N340K (neck) and K640N (motor core) of Ncd (Fig. S5) destabilize the neck/motor core interaction, resulting in the neck being positioned unstably toward either the plus or minus end (21); thus the motor moves toward either the plus or minus end of the microtubules (22). These residues are highly conserved among the kinesin-14 family, including within the KAC proteins (Figs. S5 and S6). We constructed transgenic *kac1kac2* plants expressing the corresponding mutant KAC1 (N134K[NK] and K426N[KN]) under the control of the native *KAC1* promoter and UTRs. Mutant KAC1 proteins accumulated at a drastically reduced level (Fig. S4*D*). Microbeam experiments revealed that in the NK and KN lines, the direction of chloroplast movement in both the accumulation and avoidance responses was not altered. However, the chloroplast accumulation response was severely impaired, and the velocity of chloroplast avoidance was less than half that in wild type (Fig. S4*E*). These findings may have resulted from attenuated accumulation of mutant KAC1 proteins. An unstable neck/motor core interaction also may make KAC1 unstable in vivo.

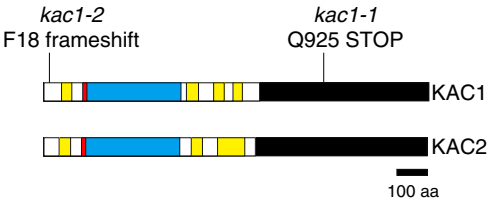

Fig. 2. KAC1 and KAC2 protein structure. Yellow: coiled-coil domain, red: neck domain, blue: motor core domain, black: conserved C-terminal region. Mutation sites of *kac1-1* and *kac1-2* mutants are indicated.

***kac* Mutants Were Defective in cp-Actin Filament Accumulation.** In petiole cells of the lines expressing GFP-mouse talin in the *kac1, kac2*, and *kac1kac2* mutant backgrounds, cp-actin filament dynamics were analyzed (Fig. 3 and Movie S7, Movie S8, Movie S9, Movie S10, Movie S11, and Movie S12). In stationary chloroplasts of wild-type and *kac2* mutant plants, cp-actin filaments were found mostly around the chloroplast periphery (Fig. 3*A*). However, significantly fewer cp-actin filaments were observed in *kac1* chloroplasts (Fig. 3 *B* and *D*), and none was found in *kac1kac2* chloroplasts (Fig. 3 *C* and *D*), similar to the *chup1* mutant that also lacked cp-actin filaments (7). Biased cp-actin filaments appearing at the front half of moving chloroplasts were found in wild-type and *kac2* mutant chloroplasts during the accumulation response toward weak blue light (Fig. 3*E*). However, in *kac1* plants under weak light, the biased cp-actin filaments were not evident, and the accumulation response was not induced (Fig. 3*F*). In response to a strong blue light microbeam, cp-actin filaments in wild-type and *kac2* chloroplasts initially disappeared but then reappeared gradually at the front half of moving chloroplasts before and during the avoidance movement (Fig. 3*G* and Movie S2) (7). This strong light-dependent disappearance of cp-actin filaments occurred normally in the *kac1* mutant, but biased cp-actin filament formation was diminished compared with that of the wild type (Fig. 3*H* and Movie S8). When the avoidance response was induced by continuous irradiation with blue GFP excitation light, less biased cp-actin filament formation and reduced avoidance were notably detected in *kac1* mutant as compared with the wild type (Movie S9 and Movie S10). In *kac1kac2*, neither cp-actin accumulation nor chloroplast avoidance occurred (Fig. 3*I*, Movie S11 and Movie S12). In wild-type plants, the difference in GFP fluorescence intensities between the front and rear halves of chloroplasts, indicative of different amounts of cp-actin filaments, showed a positive correlation with the velocity of chloroplast avoidance movement (Fig. 3*J*) (7). However, this correlation was severely attenuated in the *kac1* mutant and could not be detected in the *kac1kac2* mutant (Fig. 3*J*), possibly because of slow avoidance movement and nondirectional spontaneous rapid movements caused by a decrease in or lack of cp-actin filament accumulation, respectively.

Defective cp-Actin Filament Accumulation in *kac* Mutants Was Correlated with Impaired Chloroplast Attachment to the Plasma Membrane. Because cp-actin filaments localize to the plasma membrane side of chloroplasts and help anchor chloroplasts to the plasma membrane (7), *kac* mutants may be deficient in chloroplast anchoring. Unlike the wild type, chloroplasts in the *kac1* single mutant and, especially, in the *kac1kac2* double mutant were partially aggregated in petiole cells (Fig. 4*A*, Movie S13 and Movie S14). Close examination revealed that some chloroplasts aggregated around the nucleus in the *kac1kac2* double mutant (Fig. 4*A*). Without blue light irradiation, chloroplasts were stationary with small Brownian-like movements in the wild type but showed slightly higher motility in *kac1* and *kac2* plants (Fig. 4 *B* and *C*). However, in *kac1kac2* plants, chloroplasts translocated long distances and thus were not anchored; this effect was similar to that observed in as the *chup1* mutant, which lacks cp-actin filaments (Fig. 4 *B* and *C*) (7). Furthermore, a time-lapse movie showed that aggregated chloroplasts in *kac1kac2* cells moved rapidly via cytoplasmic streaming (Movie S14). Thus, *kac* mutants are defective in chloroplast attachment to the plasma membrane, possibly because of their lack of cp-actin filaments.

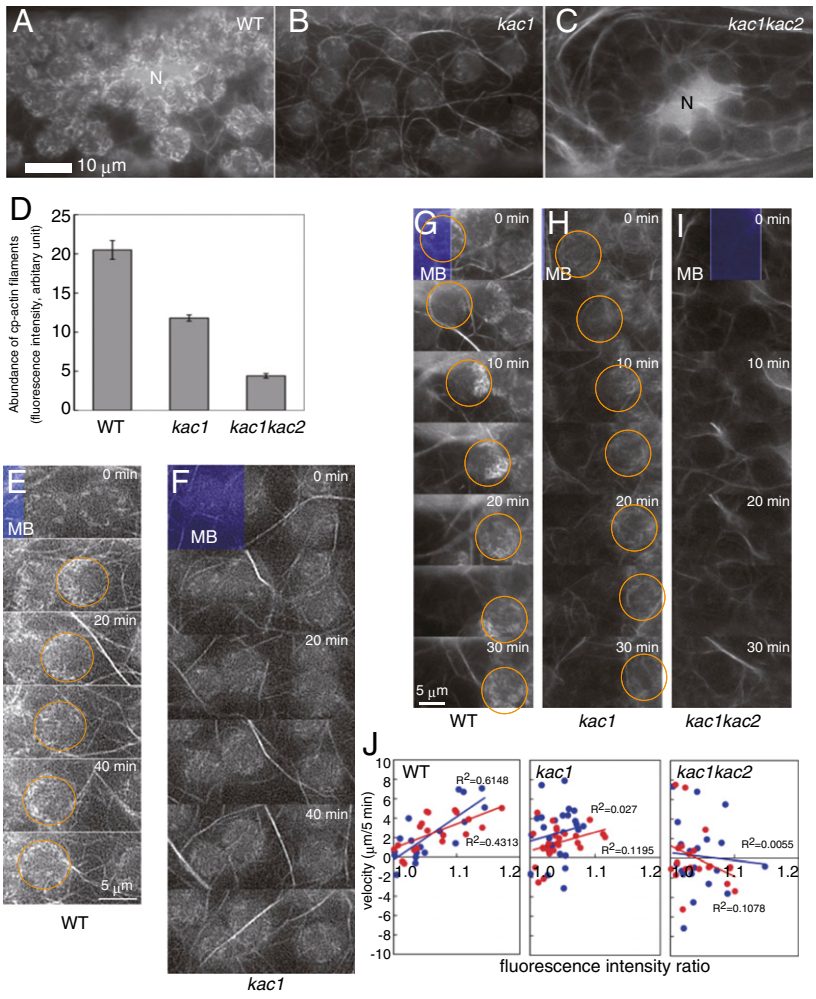

Fig. 3. Role of KAC proteins in cp-actin filament regulation. (*A–C*) cp-actin filaments on stationary chloroplasts in petiole cells. N, nucleus. (*D*) Fluorescence intensity was measured in 37–57 chloroplasts derived from three to six cells. Data are shown as mean ± SE. Because no filamentous structure was found on the chloroplasts of *kac1kac2* (*kac1-1kac2-2*), the value of *kac1kac2* represents background level of soluble GFP-talin and chlorophyll autofluorescence. *P* values determined by two-tailed Student's *t* test are *kac1* versus wild type, $P = 1.8E\text{-}10$; *kac1kac2* versus wild type, $P = 3.1E\text{-}18$; *kac1* versus *kac1kac2*, $P = 2.6E\text{-}22$. (*E–I*) Chloroplast movement was induced by continuous irradiation with a blue light microbeam (MB). Chloroplast movement and cp-actin filament dynamics were observed every 10 min under a low-intensity microbeam (3.8 μmole m^{-2} s^{-1}) (*E* and *F*) and every 5 min under a high-intensity microbeam (377 μmole m^{-2} s^{-1}) (*G–I*). Orange circles indicate moving chloroplasts. (*J*) Correlation between biased localization of cp-actin filaments (fluorescence intensity ratio) and the velocity of chloroplast avoidance during 0–5 min (bluedots) and 5–10 min (red dots) after blue microbeam irradiation. R^2, coefficient of determination.

Discussion

In this study, we identified kinesin-like KAC proteins as important regulators for cp-actin filament–mediated chloroplast movement and attachment to the plasma membrane.

The crystal structures for the motor domains of Ncd and Kar3 have already been solved (23, 24). Four conserved motifs form a nucleotide-binding pocket with topological positioning similar to that of myosins and GTPase (23, 25): N-1 (P-loop: GQTxxGKS/T), N-2 (switch I: NxxSSR), N-3 (switch II: DxxGxE), and N-4 (RxRP) (23). For a conformational change between the ATP and ADP states, kinesins likely use a mechanism similar to that of myosins and GTPases via ATP hydrolysis in switch regions (23, 26). KAC proteins of various plant species retain the highly conserved P-loop and N-4, but their switch motifs, particularly switch I, are less conserved (Figs. S5 and S6). The glycine in kinesin switch II corresponds to the highly conserved glycine in the myosin and GTPase active site, which is necessary for hydrogen bonding with γ-phosphate and for the conformational change between the nucleoside triphosphate and nucleoside diphosphate states (23, 26). The arginine in switch I and the glutamic acid in switch II form a salt bridge in kinesins similar to that of myosins (25). Mutations of these residues impaired microtubule-activated ATPase activity (26, 27). Furthermore, Kar3 switch I R598A mutant proteins showed reduced microtubule binding compared with the wild-type Kar3 (27). Notably, in seed plant KAC proteins the equivalent switch I arginine is valine, suggesting that seed plant KAC proteins cannot form the salt bridge between switch I and II motifs (Fig. S6). In rice KAC1, switch II glycine is replaced by alanine, the same substitution as the aforementioned switch II glycine mutation (26) (Fig. S6). Therefore, it is likely that vascular plant KAC proteins lack microtubule motor activity. The L12/a5 region forms a microtubule-interacting surface. Some residues for microtubule binding of human kinesin heavy chain were identified by alanine scanning (R623 and K626 in Ncd) (28). In dicot KAC proteins, the corresponding arginine and lysine are changed into acidic and nonpolar amino acids, respectively (the arginine also is different in moss KAC proteins) (Figs. S5 and S6). This amino acid changes also may be a reason why we cannot detect in vitro microtubule-binding activity of recombinant KAC motor domains. Although microtubule motor activity of KAC proteins cannot be excluded totally, the absence of microtubule-motor activity of KAC proteins is consistent with the general view that actin filaments but not microtubules are responsible for chloroplast movement in land plants.

KAC proteins were detected in both the soluble and microsomal fractions by immunoblotting. Although clear plasma membrane or organelle localization of GFP fusion proteins of KAC1 was not detected in the present study, another study revealed localization of KAC1 to the plasma membrane using immunolocalization analysis of *A. thaliana* root tips and confocal microscopy of overexpressed GFP-KCA1 (i.e., GFP-KAC1) in tobacco BY-2 cells (29). Note that GFP-KAC1 was targeted to the plasma membrane and cell plate during cell division, implicating the involvement of KAC1 in cell division as well as in chloroplast movement (29). Importantly, cp-actin filaments were localized only at the interface between the chloroplast and the

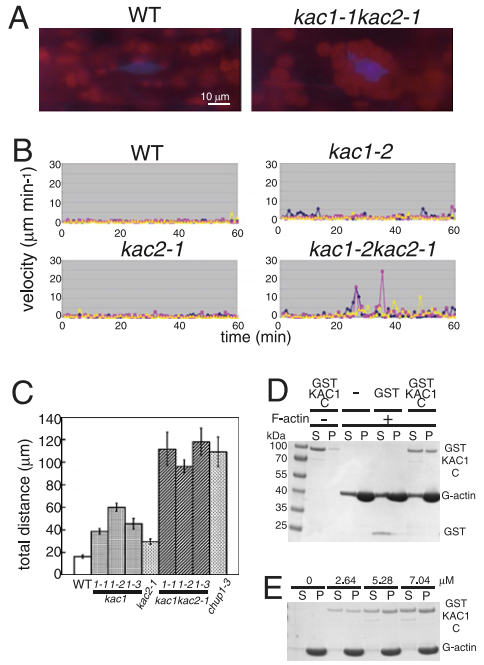

Fig. 4. Defective anchorage of chloroplasts to the plasma membrane in *kac1kac2*. (*A*) Chloroplast distribution of petiole cells in wild-type and *kac1kac2* mutant plants. Nuclei were DAPI-stained (blue). Chloroplasts were identified with red chlorophyll autofluorescence. (*B*) Chloroplast motility in wild-type and *kac* mutant lines. Each colored curve represents movement of an independent chloroplast every 1 min during 60-min incubation under red light. (*C*) Total distance traveled for 60 min in wild-type and *kac* mutant lines. For the *kac1* mutant, three alleles (*kac1-1-3*) were examined. Data represent the average of nine chloroplasts from three independent experiments. Bar indicates SE. *P* values determined by two-tailed Student's *t* test are *kac1-1* versus wild type, *P* = 3.3E-07; *kac1-2* versus wild type, *P* = 3.8E-09; *kac1-3* versus wild type, *P* = 1.8E-05; *kac2-1* versus wild type, *P* = 1.1E-04; *kac1-1kac2-1* versus wild type, *kac1-1,* or *kac2-1*, *P* = 1.0E-05, *P* = 2.0E-04, or *P* = 6.0E-05, respectively; *kac1-2kac2-1* versus wild type, *kac1-2,* or *kac2-1*, *P* = 5.4E-10, *P* = 8.2E-05, or *P* = 1.4E-08, respectively; *kac1-3kac2-1* versus wild type, *kac1-3*, or *kac2-1*, *P* = 3.4E-07, *P* = 4.3E-05, or *P* = 2.3E-06, respectively; *chup1-3* versus wild type, *P* = 2.9E-06. (*D* and *E*) Interaction between F-actin and KAC1 C-terminal domain. After incubation of 6 μM GST or GST KAC1 C-terminal domain at 25 °C for 30 min with or without 24 μM F-actin, samples were centrifuged at 228,000 × *g* for 10 min (*D*). After incubation of GST KAC1 C-terminal domain (0, 2.64, 5.28, and 7.04 μM) at 25 °C for 60 min with 24 μM F-actin, samples were centrifuged at 228,000 × *g* for 10 min. K_d = 19.7, 14.7, and 15.6 μM for 2.64, 5.28, and 7.04 μM GST-KAC1 C, respectively (*E*). Supernatant fraction (S) and precipitant fraction (P) were separated by SDS/PAGE, and the gel was stained with Coomassie brilliant blue.

plasma membrane and not at the vacuole side (7), and their accumulation was dependent on KAC proteins. Thus, it is plausible that KAC proteins localized on the plasma membrane but not in the cytoplasm may play an important role in cp-actin filament regulation and thus in chloroplast movement and attachment to the plasma membrane. Another cp-actin filament regulator, CHUP1, is localized on the chloroplast outer envelope (8–10). However, we could not detect KAC proteins in CHUP1-enriched chloroplast fractions. Although the relationship between KAC and CHUP1 in cp-actin filament regulation remains to be determined, it is obvious that further analysis of these proteins is essential for full understanding of cp-actin filament–mediated chloroplast movement.

It is interesting that kinesin-like KAC proteins mediate actin-based chloroplast movement independent of microtubules. However, there are two previously reported mechanisms in which kinesins use actin filaments for their cellular functions. First, some kinesins such as mouse KIF5B (30) and budding yeast Smy1p (31) can interact with actin-based motor myosins to use actin filaments. Notably, the function and localization of Smy1p were not perturbed by a microtubule inhibitor or by an Smy1p P-loop mutation that disturbs motor activity (32), indicating that motor activity and microtubule binding of Smy1p are dispensable for Smy1p function, similar to effects observed for KAC in this study. Although it is possible that KAC proteins may mediate chloroplast movement via direct interactions with plant myosins, recent comprehensive analyses of myosin mutant lines in *A. thaliana* and tobacco strongly suggest that myosins are not involved in chloroplast movement (33, 34). Second, some kinesins can interact directly with actin filaments (35–37). We performed a cosedimentation assay between actin filaments and several KAC recombinant proteins. We could detect an interaction between actin filaments and the KAC1 C-terminal domain (Fig. 4*D*). Both GST alone and GST-KAC1 C-terminal fusion proteins were detected in the soluble fraction without F-actin (Fig. 4*D*). However, a significant amount of the GST-KAC1 C terminus, but not GST alone, was precipitated with F-actin (K_d = ~15 μM) (Fig. 4 *D* and *E*), indicating that the KAC1 C terminus can interact with F-actin. This interaction may be necessary for cp-actin generation or maintenance in vivo. Although the precise mechanism by which KAC proteins mediate actin-based chloroplast movement is unknown, our study indicates that plants have evolved a unique kinesin-like protein-dependent mechanism for organelle movement involving specific short actin filaments but not microtubules.

Materials and Methods

Plant culture, mutant screening, and map-based cloning were performed as described previously (11). For *Agrobacterium*-mediated transformation, the T-DNA vector pBI-HI-BSKR (11) was used (Fig. S8). Chloroplast photorelocation movement was analyzed by observation of chloroplast distribution after 2 h of light irradiation (11) or by microbeam irradiation (16). Observation of actin and microtubule dynamics was performed as described previously (7).

RT-PCR, immunoblotting, and the cytoskeleton binding assay are described in *SI Text*. For GST-tagged KAC proteins, various KAC cDNAs were cloned into pGEX (Amersham Pharmacia), and recombinant proteins were purified according to the manufacturer's instructions.

Full methods and associated references are available in *SI Text*. Primers used are listed in Table S1.

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