

Multiple kinesin-14 family members drive microtubule minus end-directed transport in plant cells

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Minus end-directed cargo transport along microtubules (MTs) is exclusively driven by the molecular motor dynein in a wide variety of cell types. Interestingly, during evolution, plants have lost the genes encoding dynein; the MT motors that compensate for dynein function are unknown. Here, we show that two members of the kinesin-14 family drive minus end-directed transport in plants. Gene knockout analyses of the moss *Physcomitrella patens* revealed that the plant-specific class VI kinesin-14, KCBP, is required for minus end-directed transport of the nucleus and chloroplasts. Purified KCBP directly bound to acidic phospholipids and unidirectionally transported phospholipid liposomes along MTs in vitro. Thus, minus end-directed transport of membranous cargoes might be driven by their direct interaction with this motor protein. Newly nucleated cytoplasmic MTs represent another known cargo exhibiting minus end-directed motility, and we identified the conserved class I kinesin-14 (ATK) as the motor involved. These results suggest that kinesin-14 motors were duplicated and developed as alternative MT-based minus end-directed transporters in land plants.

Introduction

Intracellular transport along microtubule (MT) filaments plays a pivotal role in cell organization and function. Cytoplasmic dynein, a major MT-based motor in animal and fungal cells, is composed of a motor subunit (dynein heavy chain) and several associated proteins (Kardon and Vale, 2009; Hancock, 2014; Cianfrocco et al., 2015). Upon MT binding, dynein moves processively toward the MT minus end, allowing this motor protein to transport a variety of cargo, from RNA to giant organelles such as the nucleus, along MTs. Interestingly, land plants have lost most of the genes encoding dynein components, including the gene encoding the cytoplasmic dynein heavy chain (Lawrence et al., 2001). How then might plants execute minus end-directed transport?

It is generally assumed that the actomyosin system is predominantly used for cargo transport in plants (Shimmen and Yokota, 2004). However, MT-dependent transport also exists in plants and is shown to be critical in plant physiology in some instances (Kong et al., 2015; Miki et al., 2015; Nakaoka et al., 2015; Zhu et al., 2015). Little is known about which motors are responsible for transporting which cargoes. In particular, no information is available for minus end-directed transport.

Two lines of evidence are needed to demonstrate that a specific motor transports a certain intracellular cargo. First, the motor should show processive motility in vitro, wherein a single motor or a cluster of motors takes multiple steps unidirectionally along an MT. Dynein, associated with an activator protein, and many, but not all, kinesin family members fulfill this criterion (Miki et al., 2005; Cianfrocco et al., 2015). Second, cargo

motility or distribution within the cell should be perturbed when the motor protein is depleted from the cell. For instance, abnormal mitochondrial distribution in cells depleted of a processive KIF5B (kinesin-1) led to the conclusion that mitochondria are transported by this motor (Tanaka et al., 1998). Similarly, dynein-dependent transport of the nucleus was shown in a filamentous fungus by identifying a dynein mutant with altered nuclear positioning (Xiang et al., 1994). In addition to these two critical findings, colocalization of the motor and cargo constitutes supportive evidence for motor-cargo interaction (Schuster et al., 2011). Additional evidence for cargo-motor interaction is in vitro reconstitution of the transport process using pure components. For instance, kinesin-3-dependent transport of synaptic vesicles was first shown by observation of kinesin-3 mutant phenotype, followed by in vitro reconstitution of liposome transport by purified kinesin-3 (Hall and Hedgecock, 1991; Klopfenstein et al., 2002).

When searching for the minus end-directed transporter that replaces dynein function in plants, we considered members of the kinesin-14 (hereafter called kin14) family to be candidate motors, because they are known to have minus end-directed motility along MTs, unlike kinesins from other classes (Endow, 1999). However, the best-studied kin14 in animals, *Drosophila melanogaster* Ncd, is not processive; Ncd detaches from MTs after only one step along the MT (Case et al., 1997). Land plants have six kin14 subfamilies, five of which are unique to plants (Shen et al., 2012). However, all six kin14 subfamily

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Abbreviations used: KO, knockout; MT, microtubule; PC, phosphatidylcholine; PG, phosphatidylglycerol.

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members have been shown to be either nonmotile or nonprocessive in standard single-molecule motility assays (Jonsson et al., 2015; Walter et al., 2015). Interestingly, however, when KCBP-b (class VI kinesin-14) motors from *Physcomitrella patens* were artificially tetramerized or tethered on the liposome surface, they moved processively in vitro (Jonsson et al., 2015). Furthermore, such processive minus end-directed motility was observed when citrine (a GFP variant) was attached to the endogenous KCBP-b motor; punctate citrine signals representing four or more citrine molecules moved 1.0 μm on average before dissociating from MTs (Jonsson et al., 2015). Another study also reported an $\sim 0.18\text{-}\mu\text{m}$ run for KCBP in pavement cells and hypocotyl cells of *Arabidopsis thaliana* (Tian et al., 2015). These results suggest that multiple copies of KCBP on cargo surfaces are able to conduct long-distance minus end-directed cargo transport. Thus, these previous studies provided a candidate minus end-directed cargo transporter in plants. However, it remained unclear whether KCBP is the motor actually required for minus end-directed cargo transport in cells.

In the present study, we used in vivo and reconstitution approaches to test the hypothesis that KCBP directs cargo transport in plants. We focused our in vivo observation on the nucleus and newly nucleated MTs in the cytoplasm, which are, to the best of our knowledge, the only two minus end-directed transport events reported so far in the plant cytoplasm (Miki et al., 2015; Nakaoka et al., 2015). Our data indicated that *P. patens* KCBP is required for minus end-directed transport of the nucleus immediately after cell division, but not of the newborn MT. We also identified the chloroplast as another KCBP cargo. Furthermore, we reconstituted the direct binding of KCBP to acidic phospholipids and vesicle transport in vitro, and we identified class I kinesin-14 (ATK) as a motor that drives minus end-directed transport of newborn MTs. Thus, this study identified two kin14s as minus end-directed transporters in plants for the first time.

Results

KCBP is required for minus end-directed nuclear transport after cell division

To address the role of KCBP in intracellular transport, we used homologous recombination to delete *KCBP* genes one by one in a line expressing GFP-tubulin and histoneH2B-mRFP. The KCBP subfamily consists of four highly homologous genes in moss (Shen et al., 2012; Miki et al., 2014), and we obtained two independent quadruple-knockout (KO) lines (Fig. S1 A). The KO line showed cell growth retardation in protonemal tissue, indicating that vigorous moss growth requires KCBP function (Fig. S1 B). We assessed the behavior of the nucleus upon KCBP KO using long-term time-lapse microscopy. Interestingly, we observed a nuclear migration defect upon KCBP KO immediately after cell division in caulonemal apical cells, wherein daughter nuclei did not move toward the cell center but stayed near the cell plate (Fig. 1, A [0–18 min] and B; and Video 1). This phenotype was caused by the deletion of *KCBP* genes, since ectopic expression of KCBP-b rescued the phenotype (Fig. 1 B).

Because nuclear positioning is an MT-dependent process in caulonemal apical cells (Miki et al., 2015), this phenotype in the KO line was attributed to (a) lack of MT tracks around the nucleus, (b) skewed MT polarity, or (c) defects in minus end-

directed transport. To distinguish these possibilities, we imaged EB1, an MT plus-end-tracking protein, in control and KO lines after cell division (Fig. 1, C–E; and Video 2). In both lines, we observed EB1 comets around the nucleus, and the majority of them moved toward the cell plate, not to the cell tip, at this stage (Fig. 1, D and E). The data indicated that, regardless of the presence or absence of KCBP, MTs are present around the nucleus and are predominantly oriented with the plus-ends pointed toward the cell plate. Thus, the results suggest that KCBP is required for nuclear transport toward the MT minus end immediately after cell division.

We analyzed KCBP localization using spinning-disc confocal microscopy in living caulonemal apical cells, in which citrine was tagged to the N terminus of the endogenous *KCBP-b* gene (Fig. 1 F and Video 3). Citrine KCBP-b proteins did not show any particular localization during metaphase. However, immediately after mitotic chromosome segregation, citrine signals were transiently enriched near the nuclear region, most likely at the surface of the reforming nucleus (Fig. 1 F, arrowheads). The signals appeared 1–2 min after anaphase onset and were observed for the next 24 ± 5 min (\pm SD; $n = 9$). This duration matched the duration of the KCBP-dependent nuclear movement (Fig. 1 B). This finding supports the idea that KCBP is responsible for nuclear transport at this particular stage of the cell cycle.

KCBP is required for minus end-directed chloroplast motility

During cellular observations, we also noticed that chloroplasts were more apically localized in many apical cells upon KCBP KO (Fig. 2 A; chloroplasts were visible because of their strong autofluorescence). This notion was confirmed by the kymograph generated over the entire cell cycle (Fig. 2 B), as well as by the signal intensity quantification at a fixed time point (Fig. 2 C; 150 min after anaphase onset). Chloroplast positioning depends on both actin and MTs (Sato et al., 2001). To test which cytoskeletal filament is required for chloroplast positioning in our conditions, we depolymerized F-actin or MTs by treating caulonemal cells with specific inhibitors (Fig. S2). We observed a profound impact on chloroplast positioning after MT destabilization by oryzalin, whereas no clear positioning defect was observed after actin destabilization by latrunculin A. We concluded that MTs are critical for uniform distribution of the chloroplasts.

To confirm whether the abnormal distribution of chloroplasts in the KCBP KO line is caused by defects in their minus end-directed transport, we aimed to observe chloroplast dynamics at shorter time intervals (10 s). However, chloroplasts were apically enriched in the KO line in majority of the cell cycle, which hindered individual chloroplast tracking. Therefore, we focused on post-anaphase apical cells (~ 60 min after anaphase onset), in which new cell plate deposition near the nucleus reset chloroplast distribution within the cell; several individual chloroplasts were traceable for >30 min, regardless of the presence or absence of KCBP (Fig. 2, D and E; and Video 4). Our manual tracking of 108 chloroplasts in the control line indicated that chloroplasts moved back and forth, and net displacement was modest after 30 min; on average, they moved apically at a velocity similar to the tip growth speed (Figs. 2 E and S1 B). In sharp contrast, in the KO line, chloroplasts tended to translocate more apically, with suppression of basal motility

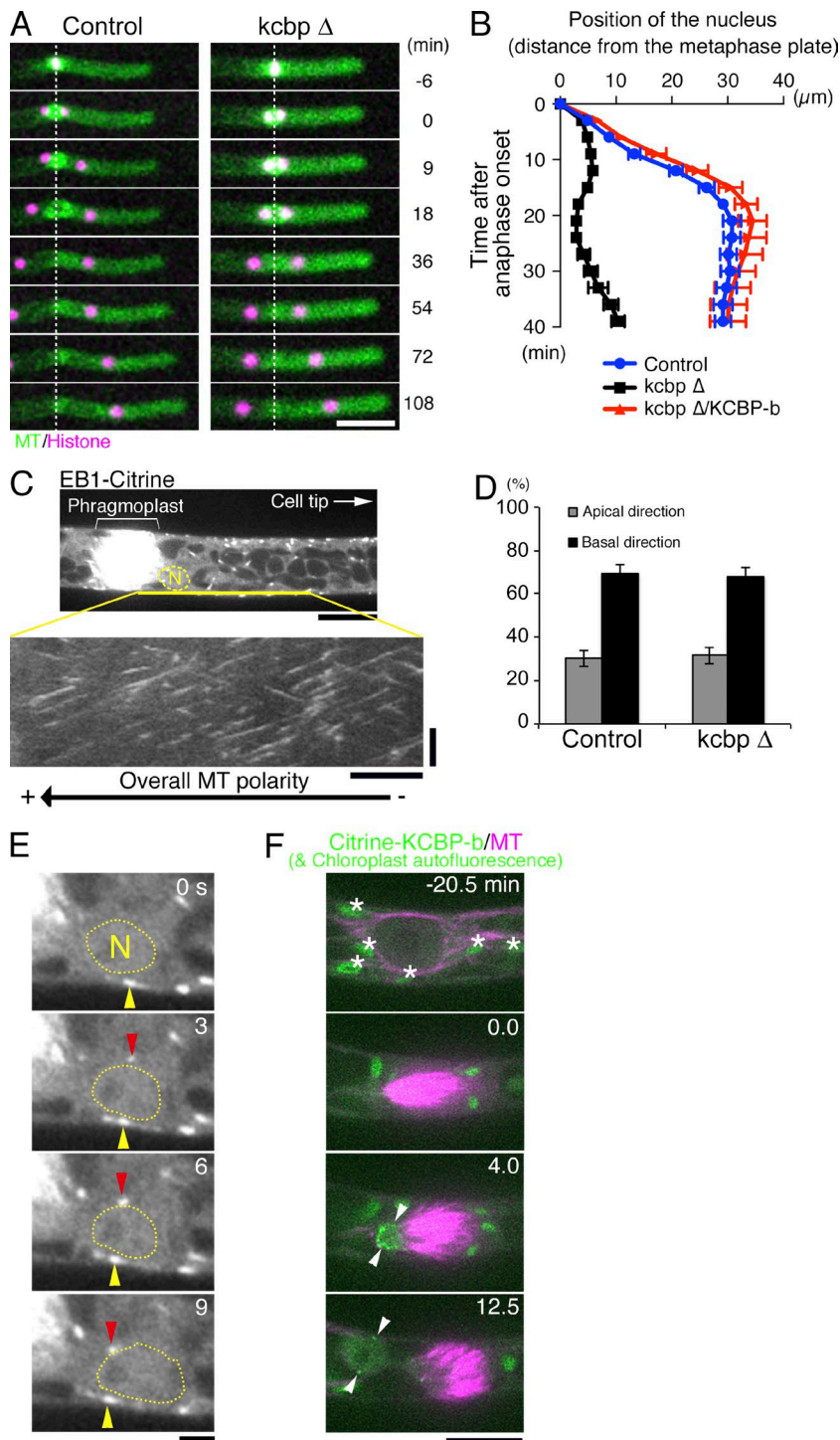


Figure 1. Defective nuclear transport in the absence of KCBP. (A) Nuclear migration defect observed immediately after cell division (0–18 min) of a KCBP KO cell. Time 0 corresponds to anaphase onset. Bar, 50 μm . White dotted lines indicate the position of the metaphase plate. (B) Position of the nucleus after anaphase onset in apical cells. Distance from the metaphase plate is plotted as the mean \pm SEM. Control and *kcbp*Δ, $n = 5$; *kcbp*Δ with ectopic KCBP-b expression, $n = 6$. (C–E) MT polarity determination by EB1 imaging. (C) Snapshot (top) and kymograph (bottom) of EB1-citrine, whose moving direction indicates MT polarity. N, nucleus. Bars: (top) 10 μm ; (horizontal) 5 μm ; (vertical in kymograph) 2 min. (D) Directionality of EB1 motility was determined using a kymograph that spanned 30 μm from the phragmoplast equator [354 [control] or 248 [KO line] signals in five cells, mean \pm SEM]. (E) EB1-citrine movement near the nucleus (arrowheads). The tip of this cell is on the right. Bar, 2 μm . (F) Localization of citrine-tagged KCBP-b proteins. Arrowheads indicate nuclear signals (the other daughter nucleus is out of focus in this video). Note that autofluorescent chloroplasts are also visible as smaller ellipsoidal structures in this image sequence (marked with asterisks in the top panel). Time 0 corresponds to anaphase onset. Bar, 10 μm .

($n = 68$). This observation explained the apical accumulation of chloroplasts in the KO line in the majority of the duration of cell cycle. At this stage, more than 60% of the EB1 signals moved toward the apical cell tip, suggesting that MTs are overall oriented in such a manner that plus-ends are facing the cell tip (Fig. 2, F–G; Hiwatashi et al., 2014). These results are consistent with the idea that, in the absence of KCBP-dependent minus end-directed motility, one or more counteracting plus-end-directed motors move chloroplasts to the tip. Thus, the minus end-directed transport defect in the absence of KCBP was not limited to the nucleus.

In vitro reconstitution of KCBP-dependent vesicle transport

KCBP has the FERM (4.1/ezrin/radixin/moesin) domain in its tail region, which is known to bind lipids (Fig. 3 A; Chishti et al., 1998). Given the identification of two membranous cargoes in cells, an interesting possibility was that KCBP might bind to membranes directly. However, in our previous study, we failed to observe direct binding of purified KCBP to a liposome made with phospholipids even after several attempts; therefore, we artificially tethered purified histidine-tagged KCBP-b to the liposome surface by introducing a Ni-NTA-conjugated lipid,

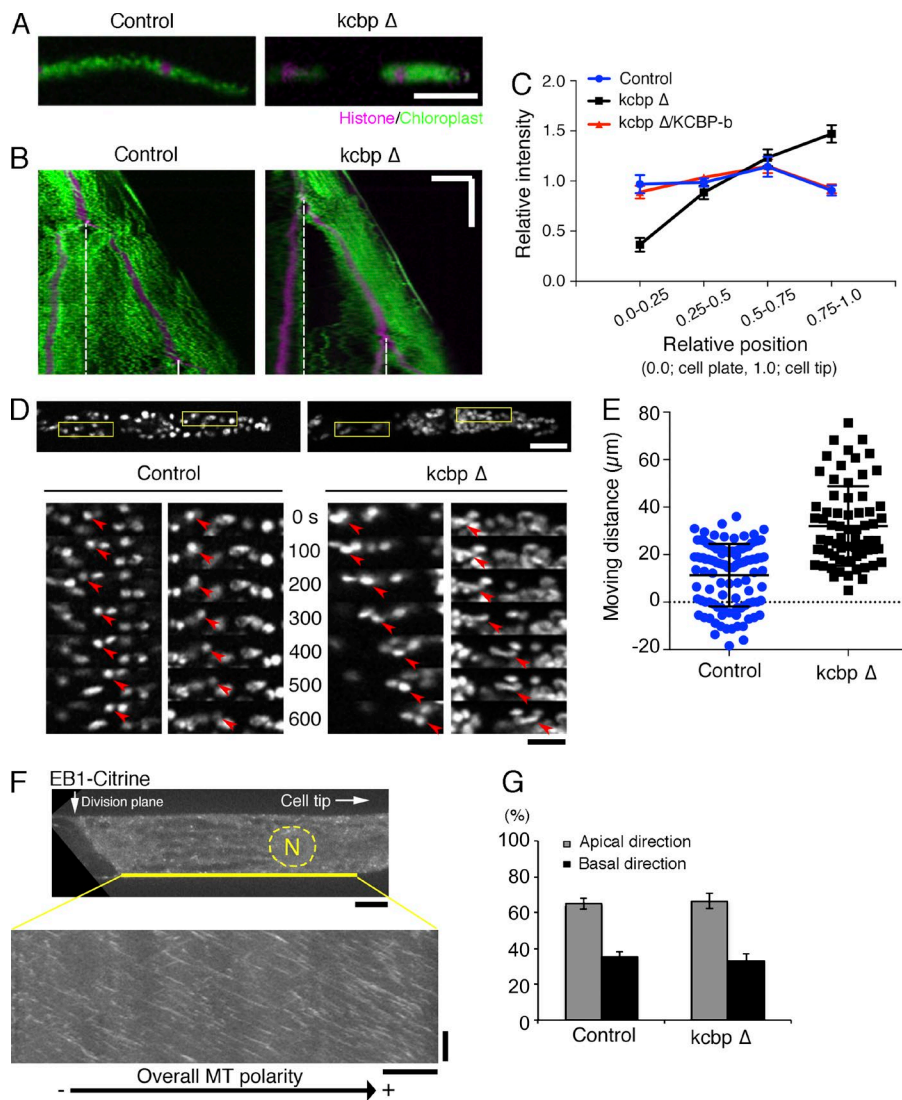


Figure 2. Abnormal chloroplast distribution in KCBP KO cells. (A) Snapshots of KCBP KO cells at 150 min after anaphase onset. green, chloroplast; magenta, histone-RFP. Bar, 50 μ m. (B) Kymograph showing chloroplast distribution along the apical–basal axis throughout the cell cycle. White dotted lines indicate the position of the metaphase plate. Bars: (horizontal) 50 μ m; (vertical) 120 min. (C) Chloroplast signal intensity was measured along cells' long axes. Each cell was divided into four regions, and the mean signal intensity within each region was divided by the mean intensity of the whole cell. Mean relative values and SEMs are shown in this graph. Control, $n = 8$; KO line, $n = 14$; KO/KCBP-b line, $n = 9$. (D) Apical translocation of chloroplasts in KCBP KO. Arrowheads indicate tracking of two chloroplasts. Bars: (top) 20 μ m; (bottom) 10 μ m. (E) Net displacement of individual chloroplasts along a cell's long axis in 30 min. Negative values were given when the chloroplast moved toward cell plate (basal direction). Mean and SD are marked (108 [control] or 68 [KO] chloroplasts in three cells; $P < 0.0001$; Mann–Whitney U test, two-tailed). (F and G) Directionality of EB1 signal motility during 60–90 min after anaphase onset (407 [control] or 235 [KO line] signals in five cells, mean \pm SEM). A snapshot and kymograph are displayed. Bars: (top) 10 μ m; (horizontal) 10 μ m; (vertical in kymograph) 2 min.

and observed liposome motility in vitro (Jonsson et al., 2015). In the present study, motivated by the defects in organelle distribution in the KCBP KO line, we revisited the liposome-binding assay for this motor.

In the previous study, we used liposomes made predominantly (90%) of phosphatidylcholine (PC), a neutral phospholipid, although the PC content of cellular membranes is typically <50% (Dewey and Barr, 1971; Schwertner and Biale, 1973; Whitman and Travis, 1985; Novitskaya et al., 2000). To better mimic cellular membranes in terms of composition of neutral and acidic phospholipids, we prepared giant hybrid liposomes composed of PC (50%) and an acidic phospholipid (50%; phosphatidylglycerol [PG], phosphatidylamine, or phosphatidylserine). Interestingly, we observed that purified GFP-KCBP-b decorated all three hybrid liposomes, but not those assembled solely with PC (Fig. 3 B; PC only and PC-PG liposome are displayed). This result explained why we failed to observe direct binding in the previous study. Liposomal binding of KCBP was tail dependent, since we did not observe GFP decoration around the PC-PG liposomes when a construct lacking a tail was used (Fig. 3 B). These results indicated that KCBP-b is capable of directly binding to acidic phospholipids. Furthermore,

it suggests that KCBP has an ability to bind to many, if not all, membranes in the cell.

To address whether KCBP can transport the phospholipid liposomes along MTs, we prepared full-length GFP-KCBP-b and smaller PC-PG liposomes that were fluorescently labeled. When they were supplied to flow chambers with MTs attached to a coverslip, unidirectional transport of the liposomes along MTs was observed (Fig. 3, C, C', and D; and Video 5). These reconstitution results demonstrated the ability of the KCBP-b motor to transport membrane vesicles via direct binding.

ATK is required for minus end-directed MT transport

Another cargo that has been demonstrated to be transported in the minus end direction in plant cells is the MT that is nucleated in a branching fashion in the cytoplasm (Nakaoka et al., 2015). Because MT association with the tail domain of *Arabidopsis* KCBP was previously shown (Tian et al., 2015), we investigated whether moss KCBP is required for branching migration of MTs. However, we observed branching MT migration in the absence of all four KCBPs (Fig. 4 A). The result indicates that yet another minus end-directed motor is involved in MT transport.

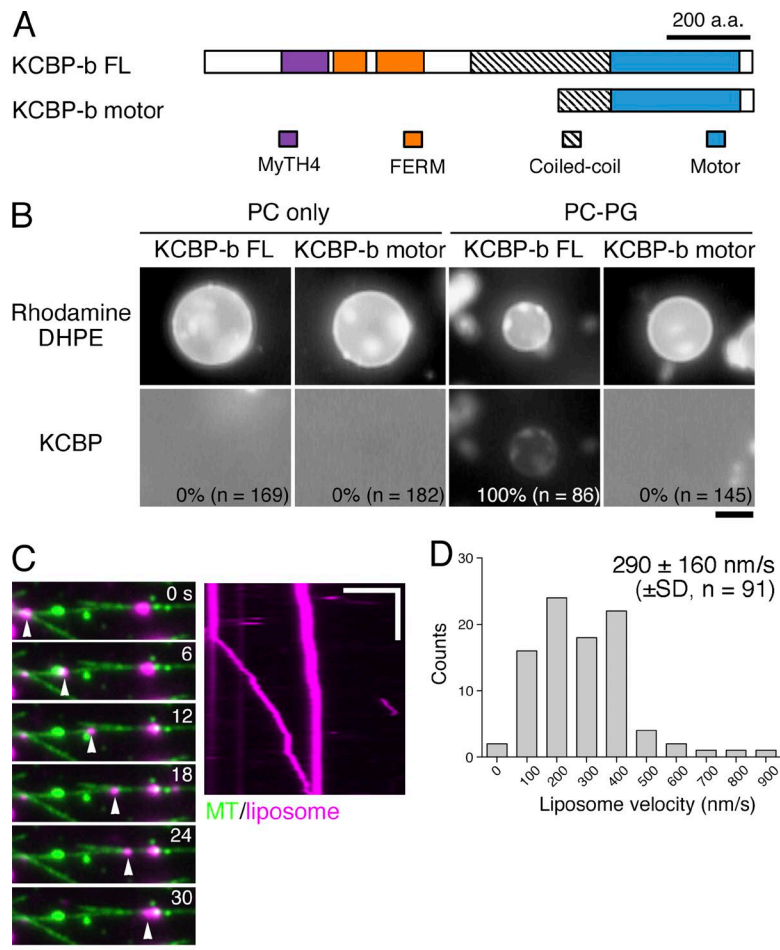


Figure 3. Reconstitution of liposome transport by KCBP. (A) Domain organization of full-length (FL) and truncated (851–1,322 aa) KCBP-b proteins used in the in vitro assay. (B) Reconstitution of KCBP-b liposome binding. Full-length GFP-KCBP-b bound to the liposome composed of PC and PG, but not the liposome made solely with PC. Full-length GFP-KCBP-b, but not truncated protein, bound to liposomes. Frequency of GFP-positive liposomes is indicated in each panel. Background GFP signals are high in three panels because of diffused GFP-kinesins. PC/KCBP-b full length, $n = 169$; PC/KCBP-b motor, $n = 182$; PC-PG/KCBP-b full length, $n = 86$; PC-PG/KCBP-b motor, $n = 145$. Bar, 5 μm . (C and D) Reconstitution of liposome transport by KCBP-b. Image sequences, kymograph, and velocity distribution are shown ($n = 91$). Arrowheads indicate a fast-moving smaller liposome, whereas a larger liposome is moving slowly. Bars: (horizontal) 2 μm ; (vertical) 10 s.

We reasoned that another kin14 family member plays such a role. The ATK/kin14-I subfamily is the only subfamily of kin14 for which orthologs are found in animals (*P. patens* ATK-a/ATK-b, *Arabidopsis* ATK1/ATK5, *Drosophila* Ncd, human HSET). Although the dimeric Ncd motor is nonprocessive, a previous study showed that artificial clustering of the Ncd motor enabled processive minus end-directed motility in vitro (Furuta et al., 2013). Similar to results observed for animal orthologs, citrine-ATK-a was predominantly localized in the nucleus during interphase and decorated MTs during mitosis (Fig. S3 A). Interestingly, spinning-disc confocal microscopy occasionally revealed punctate citrine-ATK-a signals that moved poleward at a velocity of $\sim 120 \text{ nm/s}$ ($n = 30$) in the phragmoplast (late mitotic apparatus; Fig. 4, B and C; and Video 6, right). Furthermore, surprisingly, when oblique illumination fluorescence microscopy that excludes dominant nuclear signals from the imaging field was used, we observed citrine-ATK-a spots transiently occurring on cytoplasmic MTs, and some of those spots coincided with MT branching points, where a daughter MT was migrating along a mother MT (citrine signals were detected in 10 of 13 migrating MTs; Fig. 4, D and E; and Video 6, left). MT migration correlated closely with ATK localization, as the signals were detected at a much lower frequency (17%; $n = 42$) on nonmigrating MTs that were nucleated in a branching fashion (Fig. 4 E). Thus, ATK has become a candidate minus end-directed transporter for branching MTs.

We next aimed to test whether ATK depletion affects the frequency of branching MT migration in the cytoplasm. However, we could not obtain a ATK-a and ATK-b double-KO line, even after multiple attempts, although ATK-a or ATK-b single-KO lines grew normally. We reasoned that these proteins redundantly execute an essential function in moss. Therefore, to assess the function of ATK, we selected conditional RNAi lines (Nakaoka et al., 2012), in which an inducible RNAi construct targeting the *ATK-a* gene was integrated into the genome of the ATK-b KO line. Upon RNAi induction in this line, we frequently observed frayed mitotic spindles and phragmoplasts (Fig. S3, B and C). The phenotype was consistent with the ATK family in other cell types that undergo acentrosomal cell division (Hatsumi and Endow, 1992; Chen et al., 2002; Ambrose and Cyr, 2007; Ito and Goshima, 2015). The phenotype was rescued by the expression of RNAi-insensitive *ATK-a* gene (Fig. S3 C).

Using the established RNAi line, we observed the effects of ATK depletion on branching MT migration in the cytoplasm using oblique illumination fluorescence microscopy. Consistent with our previous report (Nakaoka et al., 2015), we occasionally observed MT nucleation from the lattice of an existing MT, among which $\sim 10\%$ of the nucleated daughter MTs moved along mother MTs. However, branching migration frequency was reduced after ATK-a RNAi (Fig. 4 F). These results indicate that ATK is required for minus end-directed transport of branching MTs in the cytoplasm.

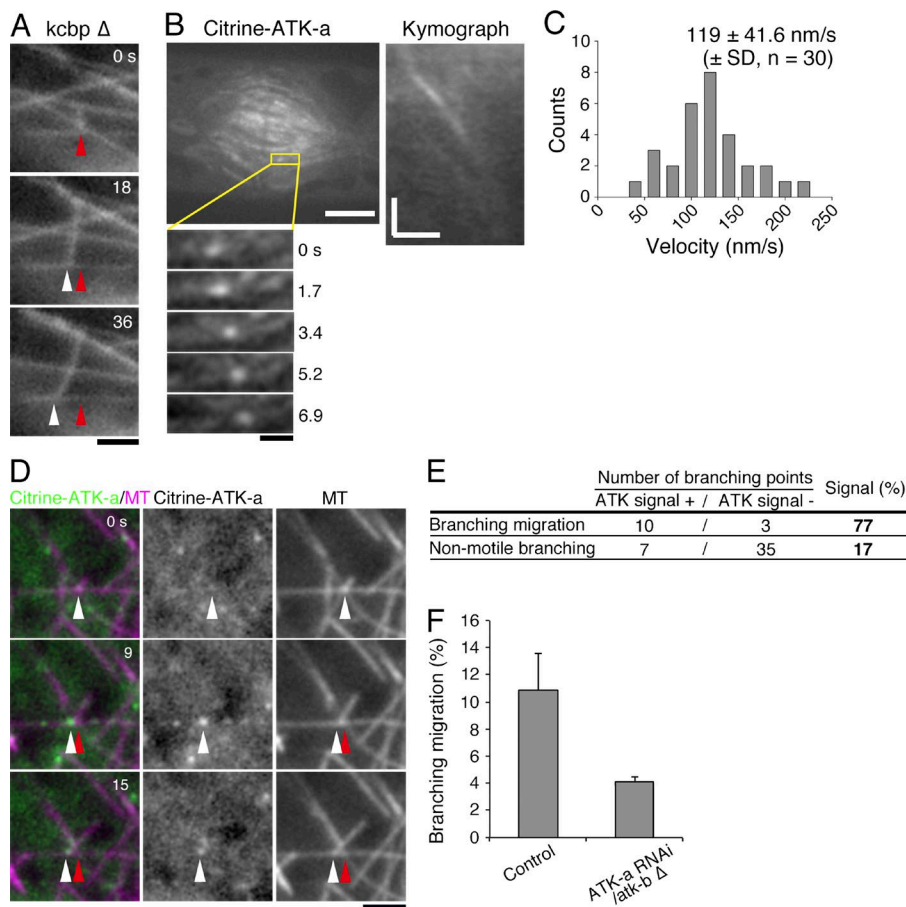


Figure 4. ATK depletion reduces branching MT migration. (A) Branching MT migration observed in the quadruple KCBP KO line. Red and white arrowheads indicate MT branching points and migrating MT, respectively. Bar, 2 μ m. (B) Poleward movement of citrine-ATK-a signals in the phragmoplast. Image sequences and kymographs are shown. Bars: (top) 5 μ m; (bottom left) 1 μ m; (kymograph) 10 μ m/2 s. (C) Velocity of citrine-ATK-a motility in the phragmoplast ($n = 30$). (D) Citrine-ATK-a signal detected at the migrating branch points (arrowheads). Bar, 2 μ m. (E) Frequency of citrine-ATK-a localization at the migrating or nonmigrating branching point. (F) Frequency of branching MT migration (per branching nucleation, \pm standard error; $n = 2$ [control *ATKb* Δ] or 3 [*ATK-a/lb* Δ RNAi] experiments). 21–37 branching MTs were identified in three cells for each experiment.

Discussion

In this study, we identified two kin14 motors as MT-based minus end-directed transporters in moss. To the best of our knowledge, these are the first cytoplasmic minus end-directed transporters identified in plants that have lost the cytoplasmic dynein complex during evolution. Among three cargoes identified, MTs and the nucleus are well-known cargoes of dynein in animals (Rusan et al., 2002; Tanenbaum et al., 2010). Minus end-directed transport has been underappreciated in plants because of the lack of dynein and the predominance of the actomyosin system for intracellular dynamics in traditional model cell types (Vale, 2003; Shimmen and Yokota, 2004). In fact, the loss of dynein in plants appeared to be partially compensated for by the development of the actomyosin system; for instance, the actin-based motor myosin XI-i is critical for nuclear motility in a few cell types in *Arabidopsis* and is essential for moss viability (Vidali et al., 2010; Tamura et al., 2013). In addition, we propose that plants developed an alternative motor-based system, wherein kin14 motors were duplicated and used as MT-based minus end-directed transporters (Fig. 5). Consistent with this notion, a previous study identified MTs, not actin, as the critical cytoskeletal filament for nuclear migration in tobacco microspores (Zonia et al., 1999). It is of interest to investigate how actomyosin- and kinesin/MT-dependent mechanisms are differentially or cooperatively used in various plant cell types.

KCBP is likely a versatile minus end-directed transporter

Several results of this study indicated that KCBP is a minus end-directed cargo transporter. First, minus end-directed motility of the nucleus and chloroplasts are suppressed upon KCBP deletion. Although loss of properly polarized MTs would lead to similar consequences, our EB1 imaging did not support this alternative possibility. Second, KCBP was enriched at the nuclear surface during the portion of the cell cycle where the nucleus exhibits minus end-directed motility. It is expected that a large cargo such as the nucleus would require the action of multiple motors for long-distance delivery (Tanenbaum et al., 2010). Whether KCBP also accumulated at the chloroplast surface remains unclear because of overwhelming autofluorescence of this organelle. Finally, phospholipid liposomes were transported in vitro by purified KCBP protein without the aid of artificial cross-linkers or any other proteins. The result suggests that KCBP cargo might not be limited to these two organelles; virtually every membranous material, from small vesicles to giant organelles, is a cargo candidate for KCBP. In addition, the tail domain of KCBP binds to MTs and actins (Tian et al., 2015); it is possible that these cytoskeletal filaments and also other proteins are transported by KCBP through direct binding or through adaptor molecules. Thus, growth retardation in moss (Fig. S1 B) or abnormal trichrome morphology in *Arabidopsis* (Oppenheimer et al., 1997; Tian et al., 2015) upon KCBP KO might result from defects in transport of various cargoes.

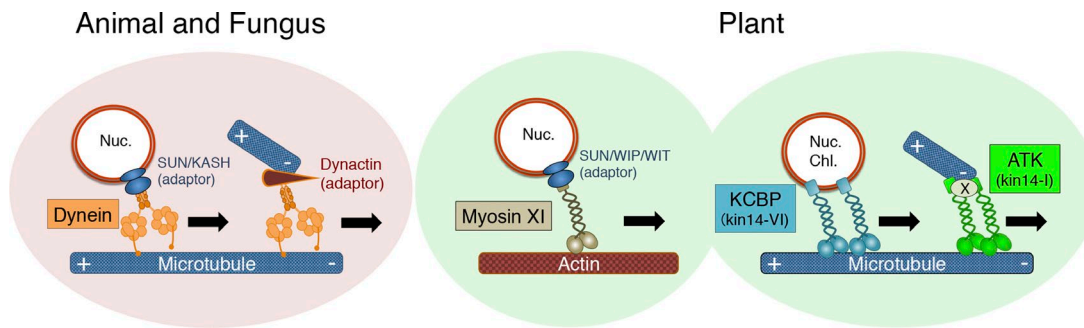


Figure 5. **Long-distance transport in plants and animals.** In animal cells, dynein is considered to be the sole transporter for long-distance, minus end-directed transport along MTs. However, land plants have lost the dynein gene. Plants have developed an actin-based transport system that uses plant-specific myosin-XI (Ueda et al., 2015), as well as the MT- and kin14-dependent mechanisms revealed in this study. Unlike dynein, at least two different kin14s are involved in the minus end-directed transport mechanism: kin14-VI/KCBP transports the nucleus, chloroplasts, and possibly other membranous cargoes via direct binding, whereas kin14-I/ATK drives MT transport along other MTs. ATK clusters at the migrating point, which might require an additional factor (depicted as factor X in this model).

Our data also suggest that KCBP activity is regulated in a cell cycle-dependent manner. We observed transient accumulation of KCBP around the nucleus, and nuclear migration defects were observed only during this period upon KCBP KO. The mechanism of KCBP regulation during the cell cycle is currently unclear. Despite the initial migration defect in the absence of KCBP, the nucleus eventually moved toward the cell center before mitosis, suggesting that redundant mechanisms are also present in moss cells (Fig. 1 A, 36–108 min). In vitro studies indicated that two other kin14 family members, kin14-II and kin14-IV, have minus end-directed motility, albeit nonprocessive, and these motors are also candidate minus end-directed transporters in cells (Jonsson et al., 2015; Walter et al., 2015). Interestingly, nuclear binding of and transport by kinesin and dynein is a regulated process in animals as well, whose molecular mechanisms are largely unclear (Tanenbaum et al., 2010; Tsai et al., 2010).

ATK is a minus end-directed MT transporter

ATK also fulfills the two criteria for a cargo transporter. First, ATK depletion from moss cells reduced the frequency of branching MT migration, namely, minus end-directed transport of newly nucleated MTs. We speculate that incomplete elimination of this event in the RNAi line is due to the presence of residual ATK protein, although it cannot be ruled out that other motor proteins are also involved in this process. Second, ATK was often concentrated at the branching point during migration in cells. Notably, the observed branching migration was reminiscent of dynein-dependent inward transport of cytoplasmic MTs during the mitotic reorganization of MT arrays in animal cells (Rusan et al., 2002). Our results suggest that plant ATK has acquired the MT-transporting activity that dynein possesses in animals.

It is unlikely that overall MT organization during interphase is grossly affected by ATK depletion, since branching MT nucleation accompanying migration constitutes only 3% of the total MT generation events in this cell type. More dominant modes are cytoplasmic nucleation, in which MTs are nucleated spontaneously in the cytoplasm without template mother MTs (56%); branching MT nucleation that does not accompany migration (30%); and MT severing (11%; Nakaoka et al., 2015). Loss of ATK, however, had a profound effect on spindle/phragmoplast coalescence. This can be partly attributed to the loss of MT bundling and sliding activities of this motor, as has been proposed for its animal counterparts (Fink et al., 2009).

However, given that we detected a cluster of GFP-ATK—a moving poleward, it is tempting to speculate that ATK-dependent branching migration takes place in the spindle/phragmoplast and assists in MT coalescence. Because MTs were crowded, particularly in the metaphase spindle, branching MT migration or minus ends of MTs could not be directly observed. Nevertheless, in support of this idea, a previous study using tobacco BY-2 cells reported poleward movement of the putative minus end marker γ -tubulin in the phragmoplast (Murata et al., 2013). Furthermore, in ATK-dependent branching migration observed in the interphase cytoplasm, the angle between daughter and mother MTs becomes shallower after migration (Nakaoka et al., 2015), suggesting that this mode of cross-linking is an excellent means to align two MTs in a parallel manner as well in the spindle/phragmoplast. Whether the ATK motor can self-cluster at MT branching points or requires additional factors, such as those that induce multimerization, remains to be determined.

Materials and methods

Moss culture, plasmid construction, gene disruption, and citrine tagging

Plasmids and primers used for protein expression, gene disruption, genotyping, and citrine tagging are listed in Table S1; moss lines (Gransden 2004 background) are listed in Table S2. Methodologies of moss culture, transformation, and transgenic line selection (citrine tagging) were previously described thoroughly (Yamada et al., 2016). In brief, we used BCDAT medium for routine culture. Transformation was performed by the standard polyethylene glycol-mediated method. Citrine tags were added to the N termini of kin14 genes via homologous recombination (drug-resistant genes were not integrated into the genome). Gene KOs were obtained by replacing an endogenous kin14 gene with a drug-resistant marker flanked by lox-P sequences. When two genes were depleted, two marker cassettes were sequentially integrated into each gene locus. For triple- or quadruple-KO selection, the existing two markers were removed by transient expression of Cre recombinase, followed by replacing the other two genes with the same markers (Cre-expressing plasmid pTN75 was a gift from M. Hasebe, National Institute for Basic Biology, Okazaki, Japan). Gene disruption was confirmed by PCR.

In vivo microscopy

The methodology for inducible RNAi was previously described thoroughly (Miki et al., 2016). For mitosis imaging after conditional ATK-a

RNAi, cells were precultured in BCD medium for 4 d, followed by RNAi induction with 1 μ M β -estradiol for 3 d. Branching migration was quantified after 10 d of RNAi induction (RNAi was less penetrant in this condition because cells had to be plated on a cellophane-coated medium for oblique illumination microscopy). Oryzalin (10 μ M; AccuStandard), latrunculin A (25 μ M; Wako Pure Chemical Industries), or control DMSO (1%) was added to protonemal cells plated on culture medium for 5–6 d. Methods for epifluorescence and spinning-disc confocal microscopy were previously described thoroughly (Yamada et al., 2016). In brief, protonemal cells were plated onto glass-bottom plates coated with BCD agar medium. Long-term imaging by a wide-field microscope (low-magnification lens) was performed with a Nikon Ti (10 \times , 0.45-NA lens, EMCCD camera Evolve [Roper]). High-resolution imaging was performed with a spinning-disc confocal microscope (TE2000 or Ti [Nikon]; 100 \times , 1.40-NA, 100, \times 1.45-NA, or 20 \times , 0.75-NA lens, CSU-X1 [Yokogawa Electric Corporation], EMCCD camera ImageEM [Hamamatsu Photonics]). Oblique illumination microscopy was performed as previously described (Jonsson et al., 2015; Nakao et al., 2015); cells were cultured in BCDAT medium and a Nikon Ti microscope with TIRF unit, a 100 \times , 1.49-NA lens, GEMINI split view (Hamamatsu Photonics), and EMCCD camera Evolve was used. Branching migration was also scored using this microscopy setup. Images were acquired every 3 s for 10 min. All imaging was performed at 24–25°C under dark conditions except for growth speed imaging, during which white light was illuminated for 100 s between image acquisitions. Microscopes were controlled by micromanager.

Protein purification

KCBP-b proteins used in this study have sequences identical to those previously reported (Jonsson et al., 2015). His-mGFP-KCBP-b (full-length) proteins were purified with Ni-NTA beads from insect Sf21 cells. The lysis buffer contained 25 mM MOPS, pH 7.0, 2 mM MgCl₂, 250 mM NaCl, 5% sucrose, 5 mM β -mercaptoethanol, 1 mM ATP, 30 mM imidazole, 1% Triton X-100, and protease inhibitors (0.5 mM PMSF and peptide cocktails [1 μ g/ml leupeptin, pepstatin, chymostatin, and aprotinin]), and the elution buffer contained 25 mM MOPS, pH 7.0, 2 mM MgCl₂, 250 mM NaCl, 5% sucrose, 5 mM β -mercaptoethanol, 1 mM ATP, and 400 mM imidazole. Truncated His-GFP-KCBP-b (851–1,322 aa) was expressed in *Escherichia coli* BL21-AI with 0.2% arabinose and 500 μ M IPTG for 18 h at 18°C. Harvested cells were lysed with the Advanced Digital Sonifier D450 (Branson) in the lysis buffer described above, followed by purification using Ni-NTA beads. Imidazole was removed at the final step by dialysis using PD MiniTrap G-25 column (GE Healthcare) with the elution buffer. Proteins were flash frozen and stored at –80°C.

In vitro liposome binding/motility assay

To prepare and observe giant liposomes, a mixture of phospholipids, egg (powder) PC (Avanti Polar Lipids, Inc.), egg (powder) PG (Avanti Polar Lipids, Inc.), and rhodamine DHPE (50:50:0.3, mol/mol; Molecular Probes), was dissolved in chloroform, dried under a constant stream of N₂ gas, and desiccated in a vacuum for at least 2 h to produce dried lipid film (Tanaka-Takiguchi et al., 2013). This film was then hydrated with 420 mM sucrose at 50°C for 120 min to obtain a liposome suspension. For the kinesin-liposome binding assay, the liposome suspension was diluted 20-fold with 1 \times assay buffer (25 mM MOPS, pH 7.0, 2 mM MgCl₂, 75 mM KCl, and 1 mM EGTA; final 40 μ M lipids), mixed with each kinesin sample (400 nM), and then observed by fluorescence microscopy (BX60 microscope [Olympus] with 100 \times , 0.75-NA lens and WAT-910HX CCD camera [Watec]). Transport assays were performed by a previously described motility assay protocol (Jonsson et al., 2015) with some modifications. Importantly, KCBP

protein was directly bound to liposomes in the current study, whereas in the previous study, histidine-tagged KCBP was bound to liposomes that contained Ni-NTA-conjugated lipids (Jonsson et al., 2015). Anti-biotin (1–5% in 1 \times MRB80; Invitrogen) was added to a flow chamber made with silanized coverslips and allowed to incubate for 5 min. The flow chamber was washed with 1 \times assay buffer and supplied with 1% Pluronic F127 (another 2- to 3-min incubation). The flow chamber was then washed with the assay buffer, followed by 5-min incubation with labeled MTs (10% Alexa Fluor 647-labeled tubulin and 10% biotin-labeled tubulin) and 40 μ M taxol. The flow chamber was washed with the assay buffer (with 2 mM ATP, 20 μ M taxol, 0.1% methylcellulose, 0.5 mg/ml casein, and oxygen scavenger system [50 mM glucose, 400 μ g/ml glucose-oxidase, 200 μ g/ml catalase, and 4 mM DTT]) and 1 μ M liposomes were added. Liposomes and kinesin solutions were mixed immediately before imaging. The liposome solution was extruded through a polycarbonate filter with 200-nm pore size using a mini-extruder (Avanti Polar Lipids, Inc.), and the motors were subjected to MT binding and release to select active motors.

Data analysis

To quantify the cell growth rate, we acquired cell images every 3 min for 10 h. Kymographs were then generated for tip-growing caulonemal cells by using ImageJ, and the tip growth rate was measured. In some cases, growth velocity decreased from the middle of the image sequences, perhaps because of shortage of light-dependent energy; in such cases, initial growth speed was measured. Quantification of the nuclear movement after cell division was performed for caulonemal apical cells. Images were acquired every 3 min for 10 h under dark conditions, and kymographs were generated. The distance from the metaphase plate to the nuclear center was measured after generating kymographs. To analyze MT polarity in post-anaphase cells, EB1-citrine or EB1-mCherry was imaged every 3 s using the spinning-disc confocal microscope (Kosetsu et al., 2013). Directionality of the EB1 motility was determined after generating kymographs along the cell's long axis, which spanned 30 μ m from the cell plate. MT polarity in interphase was determined in a similar way. The velocity of citrine-ATK-a motility in the phragmoplast was also quantified based on kymographs. Images were acquired at the phragmoplast surface every 0.8 s. Citrine-ATK-a/atk-b Δ line was used for this assay. ATK localization during interphase was analyzed based on images acquired every 3 s with oblique illumination fluorescence microscopy. In analyzing ATK localization at nonmotile branching points, we focused on the branch at which daughter MT length was less than 3 μ m, because we observed that eight of nine migrating daughter MTs were <3 μ m in length. To quantify chloroplast distribution, the intensity of chloroplast autofluorescence was measured (visualized with 640-nm laser). Images were acquired every 3 min for 10 h under dark conditions, and the chloroplast signal intensity was measured along cells' long axes by drawing a 1-pixel-wide line using ImageJ. Each cell was divided into four regions, and the mean signal intensity within each region was divided by the average intensity of the whole cell. For the liposome-kinesin binding experiment, we randomly selected liposomes with a diameter of more than 2 μ m and checked whether the liposome had ring-shaped GFP signal, which represented membrane association. When several liposomes formed an aggregate, we counted the aggregate as one liposome.

Statistics

The Mann–Whitney *U* test (two-tailed) was used.

Accession numbers

Sequence data used in this article can be found in the Phytozome database by the following accession numbers: *KCBP-a*, Pp3c15_3730; *KCBP-b*,

Pp3c9_4530; *KCBP-c*, Pp3c2_29920; *KCBP-d*, Pp3c11_5890; *ATK-a*, Pp3c7_11530; and *ATK-b*, Pp3c11_17850.

Online supplemental material

Fig. S1 shows growth retardation by deleting all four *KCBP* genes. Fig. S2 shows that chloroplast positioning requires MT cytoskeleton. Fig. S3 shows that *ATK/kin14-I* is required for spindle coalescence. Video 1 shows that nuclear migration immediately after cell division is defective in the quadruple *KCBP* KO line. Video 2 shows EB1-citrine imaging during telophase. Video 3 shows localization of citrine-*KCBP-b*. Video 4 shows that chloroplast movement is defective in *KCBP* KO. Video 5 shows liposome transport by *KCBP* in vitro. Video 6 shows motility of citrine-*ATK-a* signals during branching MT migration and in the phragmoplast.

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