

Myosin XI-K Is Required for Rapid Trafficking of Golgi Stacks, Peroxisomes, and Mitochondria in Leaf Cells of *Nicotiana benthamiana*^{1[W][OA]}

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A prominent feature of plant cells is the rapid, incessant movement of the organelles traditionally defined as cytoplasmic streaming and attributed to actomyosin motility. We sequenced six complete *Nicotiana benthamiana* cDNAs that encode class XI and class VIII myosins. Phylogenetic analysis indicates that these two classes of myosins diverged prior to the radiation of green algae and land plants from a common ancestor and that the common ancestor of land plants likely possessed at least seven myosins. We further report here that movement of Golgi stacks, mitochondria, and peroxisomes in the leaf cells of *N. benthamiana* is mediated mainly by myosin XI-K. Suppression of myosin XI-K function using dominant negative inhibition or RNA interference dramatically reduced movement of each of these organelles. When similar approaches were used to inhibit functions of myosin XI-2 or XI-F, only moderate to marginal effects were observed. Organelle trafficking was virtually unaffected in response to inhibition of each of the three class VIII myosins. Interestingly, none of the tested six myosins appears to be involved in light-induced movements of chloroplasts. Taken together, these data strongly suggest that myosin XI-K has a major role in trafficking of Golgi stacks, mitochondria, and peroxisomes, whereas myosins XI-2 and XI-F might perform accessory functions in this process. In addition, our analysis of thousands of individual organelles revealed independent movement patterns for Golgi stacks, mitochondria, and peroxisomes, indicating that the notion of coordinated cytoplasmic streaming is not generally applicable to higher plants.

Membrane-bounded organelles are essential for the function of any eukaryotic cell. Their inheritance and position within the cell are tightly regulated throughout the cell cycle (Pruyne et al., 2004; Weisman, 2006). The required translocations of organelles generally depend on cytoskeletal motility systems (Vale, 2003; Boldogh and Pon, 2006). Although plant cells are less motile than animal cells, they exhibit extensive intracellular dynamics that involve repositioning of the nucleus, reshaping of the vacuole, and rapid trafficking of the Golgi stacks, mitochondria, and peroxisomes (Shimmen and Yokota, 2004; Wada and Suetsugu, 2004; Matheson et al., 2006). This trafficking was originally characterized in filamentous algae where the continuous directional flow of cytoplasm is believed to carry

the organelles along the cell wall-associated files of chloroplasts (Shimmen and Yokota, 1994). High velocity organelle movement in angiosperms is also traditionally attributed to such directional flow, known as cytoplasmic streaming (Shimmen and Yokota, 2004; Shimmen, 2007). Although the functional significance of organelle motility in nondividing cells is not understood, its occurrence throughout the plant kingdom suggests an important physiological role. In addition, chloroplasts are engaged in much slower light-induced movements (Wada et al., 2003). It was found that destabilization of actin microfilaments by drugs abolishes organelle trafficking and inheritance, as well as chloroplast and nucleus repositioning (Boevink et al., 1998; Nebenfuhr et al., 1999; Mano et al., 2002; Hoffmann and Nebenfuhr, 2004; Sheahan et al., 2004; Kim et al., 2005; Hanton et al., 2006).

The actomyosin motility system also has been implicated in translocation of plant viruses and protein targeting to plasmodesmata (Boevink and Oparka, 2005; Prokhnevsky et al., 2005; Lucas, 2006; Wright et al., 2007). Thus, transport of organelles and macromolecular complexes in plants heavily relies on the actin cytoskeleton and is likely empowered by the associated myosin motors. However, direct evidence in support of this hypothesis is lacking, and the identity of the myosin(s) involved remains unknown.

Myosins are conserved molecular motors of eukaryotes (Vale, 2003). Evolution of myosins involved a

¹ This work was supported in part by the National Institutes of Health (grant no. GM053190 to V.V.D.) and by the Vaadia/U.S.-Israel Binational Agricultural Research and Development Fund (postdoctoral fellowship award no. F1-354-2004 to D.A.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.107.113647

complex history of lineage-specific duplications, such that the myosin superfamily is subdivided into as many as 37 classes (Richards and Cavalier-Smith, 2005; Foth et al., 2006). The angiosperms have two classes of myosins, VIII and XI, with the latter being the largest. Plant class XI myosins share a close relationship with the class V myosins found in metazoa and fungi (Foth et al., 2006; Li and Nebenfuhr, 2007). Class V myosins are processive molecular motors that function in organelle and vesicle transport and partitioning during cell division, mitotic spindle positioning, localization of mRNA, and establishment of cell polarity (Pruyne et al., 2004; Sellers and Veigel, 2006; Desnos et al., 2007). Interestingly, these diverse functions that require interactions with multiple cargoes can be performed by a single class V myosin, such as yeast (*Saccharomyces cerevisiae*) Myo2p (Pashkova et al., 2006). Typically, fungi and metazoa possess one to three class V myosins, whereas plants have a dozen or so related class XI myosins, which suggests a greater functional diversification.

Similarly to other myosins, class V and class XI myosins possess an N-terminal head or motor domain that hydrolyzes ATP and binds actin microfilaments (Vale, 2003). The C-terminal myosin tails harbor IQ motifs that are involved in interactions with calmodulin, coiled-coil motifs responsible for dimerization, and globular tail domains (GTDs). The recent determination of its three-dimensional structure revealed that GTD of the yeast class V myosin contains two subdomains formed by multiple α -helices (Pashkova et al., 2006). Each of these subdomains contains receptors that recognize distinct cargoes.

The genome of the reference plant *Arabidopsis thaliana* encodes 13 class XI and four class VIII myosins (Reddy and Day, 2001), of which only a few have been studied in any detail (Lee and Liu, 2004). The fastest, among known molecular motors, processive motion supported by myosin XI-2 ortholog from *Nicotiana benthamiana* suggested that it might play a role in cytoplasmic streaming (Tominaga et al., 2003). It was also reported that inactivation of the XI-2 (same as MYA2) gene in *Arabidopsis* resulted in a slower movement of certain vesicles and severe developmental abnormalities (Holweg and Nick, 2004). However, because two independent studies, including an accompanying article (Peremyslov et al., 2008), failed to reproduce the latter phenotype (Hashimoto et al., 2005), its significance remains unclear. Recent publications demonstrated that GTDs of class XI myosins are structurally and functionally similar to GTDs of yeast class V myosins and are involved in binding the various organelles as their cargoes (Li and Nebenfuhr, 2007; Reisen and Hanson, 2007). In addition, *Arabidopsis* myosin XI-K was implicated in the root hair growth (Ojangu et al., 2007).

Using overexpression of dominant negative myosin mutants and RNA interference (RNAi), we show that the class XI myosin XI-K plays a major role in the movement of Golgi stacks, mitochondria, and peroxisomes in the leaf cells. Our analyses of organelle traf-

ficking patterns suggest a revision of the general notion of continuous cytoplasmic streaming in higher plants.

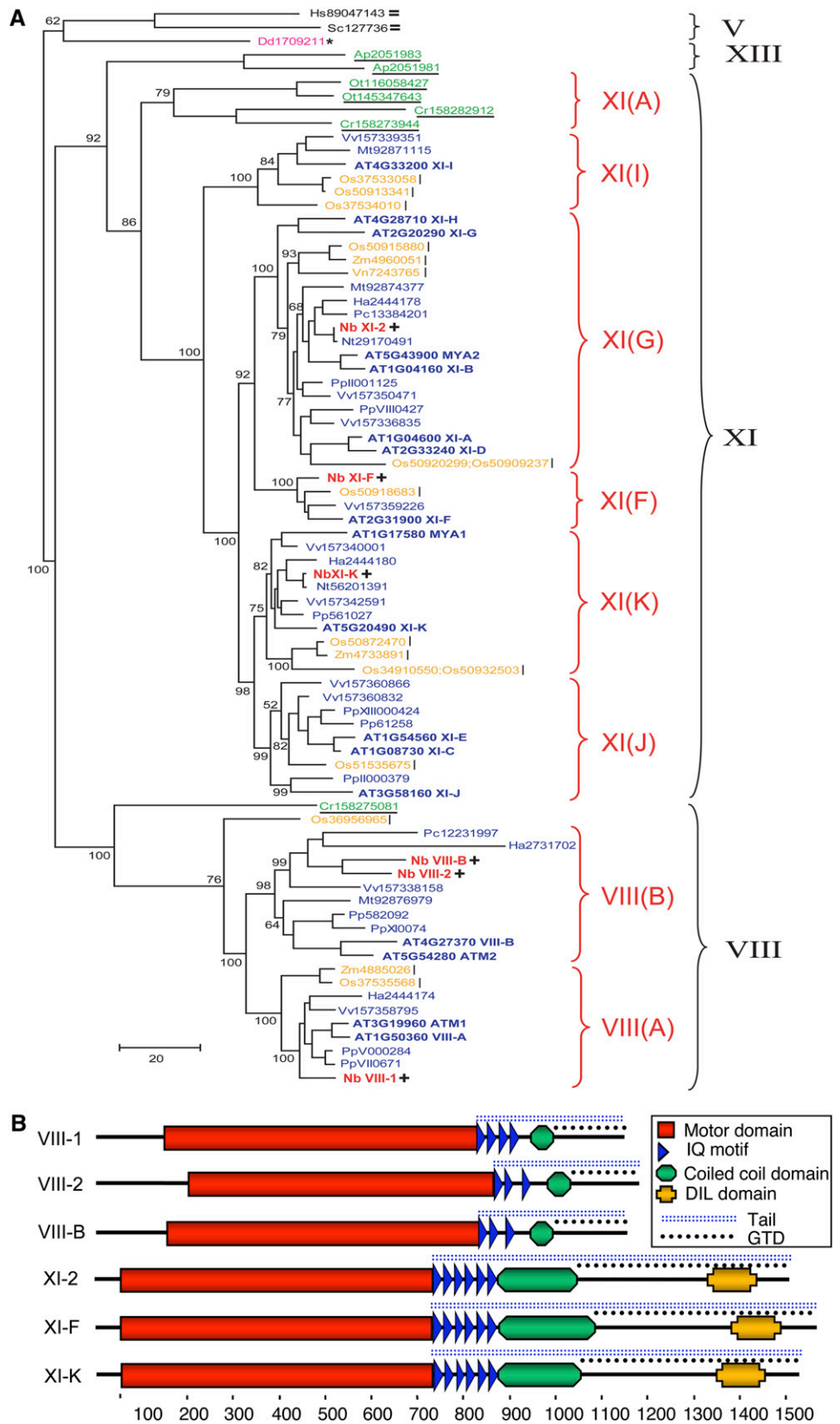
RESULTS

Isolation of Myosin cDNAs from *N. benthamiana* and Phylogenetic Analysis of Plant and Algal Myosins

To enable the analysis of organelle movement in *N. benthamiana*, a system that provides convenient assays for transient protein expression, RNAi, and organelle tracking, the nucleotide sequences of six distinct myosin cDNAs were determined. These sequences complement the current, increasingly representative set of plant and algal myosin sequences that includes the full repertoire of genes from four complete flowering plant genomes (*Arabidopsis*, rice [*Oryza sativa*], poplar [*Populus* spp.], and grapevine [*Vitis vinifera*]), two genomes of unicellular green algae (*Chlamydomonas reinhardtii* and *Ostreococcus lucimarinus*), and several other myosins from various plants (Supplemental Table S1). To ascertain the phylogenetic affinities of each of the sequenced *N. benthamiana* myosins and to elucidate salient features of myosin evolution in green plants and algae, we performed a detailed phylogenetic analysis. A maximum-likelihood tree of class VIII, XI, and XIII myosins was constructed using three sequences from myosin class V as an outgroup (see Supplemental Fig. S1 for corresponding multiple alignment). The topology of the resulting tree shown in Figure 1A is generally compatible with the conclusions of a recent study of myosin evolution (Foth et al., 2006). Specifically, the branch that includes two previously described class XIII myosins from the green alga *Acetabularia peniculus* is monophyletic with type XI myosins of Magnoliophyta, and together class XI and class XIII myosins form a sister group to class VIII myosins of Magnoliophyta (Fig. 1A). Two of the three myosins of *C. reinhardtii* and both myosins of *O. lucimarinus* belong to class XI, whereas the third myosin of *C. reinhardtii* belongs to class VIII (Fig. 1A) and, similarly to class VIII myosins of land plants, lacks the dilute domain that is present in all class V, XI, and XIII myosins (Fig. 1B). These findings suggest that the divergence of class VIII and class XI myosins antedates the last common ancestor of land plants and green algae; given that *O. lucimarinus* has a compact, probably highly reduced genome, it appears most likely that the gene for the class VIII myosin was lost in the *Ostreococcus* lineage.

The amended data set analyzed here provided for a better resolution of the scenario of myosin evolution in land plants. We found that class VIII myosins split into two distinct lineages [VIII(A) and VIII(B)], whereas class XI myosins split into five lineages [XI(I), XI(G), XI(F), XI(K), and XI(J); the designations for the clades are taken from representatives from *Arabidopsis*]. Each of these clades is strongly supported by bootstrap analysis, and all except VIII(B) include both dicot and monocot species. Conceivably, the basal position of

Figure 1. A, Phylogenetic tree for the motor domain of plant myosins. Each terminal node of the tree is labeled by the two-letter abbreviation of the corresponding species name and the numeric GenBank identifier (GI). For Arabidopsis, identifiers assigned previously (Reddy and Day, 2001) are also indicated. Identical sequences encoded in distinct loci in rice are separated by semicolon. Coding is as follows: dicots, dark blue (unmarked) except for Arabidopsis marked in bold and *N. benthamiana* marked in red and [+]; monocots, yellow and [|]; green algae, green and underlined; human and yeast, black and [=]; *Dictyostelium discoideum*, magenta and [*]. Abbreviations: Ap, *A. peniculus*; At, Arabidopsis; Cr, *C. reinhardtii*; Dd, *D. discoideum*; Ha, *Helianthus annuus*; Hs, *Homo sapiens*; Mt, *Medicago truncatula*; Nb, *N. benthamiana*; Nt, *Nicotiana tabacum*; Os, rice; Ot, *O. lucimarinus*; Pc, *Petroselinum crispum*; Sc, yeast; Vn, *Vallisneria natans*; Vv, grapevine; Zm, *Zea mays*. PpXIII000424, PpII001125, PpII000379, PpVII0671, PpVIII0427, PpXI0074, PpV000284, Pp582092, and Pp61258 are sequences from draft genome of *Populus trichocarpa* taken from the JGI Web site (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>). For selected major branches, bootstrap probabilities (%) are shown. B, Domain structure of the *N. benthamiana* myosins. Dotted lines correspond to truncated myosin variants used for over-expression. The scale below the diagram shows the numbers of amino acid residues.



one of the rice myosins reflects accelerated evolution, and this myosin actually might belong in group VIII(B). Thus, at least seven lineages of class XI and VIII myosins appear to have been represented already in the common ancestor of Magnoliophyta, implying their early functional specialization. Duplication of myosin genes during plant evolution seems to have been quite prolific, in contrast to the near lack of such duplications in the currently available algal genomes. In addition to the duplications that occurred prior to the divergence of monocots and dicots, each of these branches has many lineage-specific paralogs (Fig. 1A).

Phylogenetic analysis informed classification of *N. benthamiana* myosins that belong to five out of the seven identified groups. These myosins were designated with the letters or digits previously assigned to the most closely related Arabidopsis myosins (Reddy and Day, 2001): XI-2 (same as MYA2), XI-F, XI-K, VIII-1 (same as ATM1), VIII-2 (same as ATM2), and VIII-B.

Myosin XI-K Tails Interfere with Organelle Movement in *N. benthamiana*

Because the attachment of the cargo such as organelle is mediated by myosin tails, overexpression of headless tails is expected to interfere with the tail-binding capacity of the organelles and inhibit their transport. Alternatively, free tails might interact with the heads of corresponding myosins, thus reducing the motor activity (Krementsov et al., 2004). The dominant negative inhibition strategy was employed to determine the roles of each of the six *N. benthamiana* myosins in the trafficking of Golgi stacks, peroxisomes, and mitochondria. The corresponding hemagglutinin (HA)-tagged myosin tails or shorter GTDs (Fig. 1B) were transiently expressed in the leaves using agroinfiltration (Fig. 2A). Such expression did not affect the actin microfilament architecture in a detectable way (Fig. 2B, top row). The organization of the endoplasmic reticulum (ER) network that, in the plant cells, is supported by actin microfilaments was also unaffected (Fig. 2B, bottom row).

The Golgi stacks and peroxisomes were simultaneously visualized in the leaf epidermal cells using transient expression of the Golgi-targeted yellow fluorescent protein (YFP) and the peroxisome-targeted mCherry (Fig. 3A, top row, green and magenta, respectively). The patterns, velocities, and displacement rate of at least 400 individual organelles in the presence of each coexpressed myosin tail were determined using Volocity software. It should be emphasized that over 99% of the cells in the infiltrated areas expressed both organelle markers. This result strongly suggested that the vast majority of these cells also coexpressed the myosin tails as appropriate for each experimental variant (see "Materials and Methods" for details).

It was found that the tails of myosins XI-2, XI-F, VIII-1, VIII-2, or VIII-B did not significantly affect the Golgi trafficking patterns, mean velocity (Fig. 3, A and B; Supplemental Movies S1 and S2), or displacement rate (Supplemental Table S2). In contrast, overexpression of

myosin XI-K tail nearly halted Golgi trafficking (Fig. 3A, column XI-K; Supplemental Movie S1), resulting in an approximately 10-fold reduction of the mean velocity (Fig. 3B) and displacement rate (Supplemental Table S2). It should be mentioned that the residual low velocity was largely due to oscillations rather than processive movement of organelles (Fig. 3A, bottom row; Supplemental Movie S1). The mean distance traveled by Golgi stacks over the observation period in the presence of myosin XI-K tails was only approximately 0.6 μm compared to approximately 30 μm for the control (Supplemental Table S2). Such dramatic reduction in organelle trafficking was observed in virtually all cells in the infiltrated leaf area supporting the uniformity of the myosin tail expression levels among individual cells.

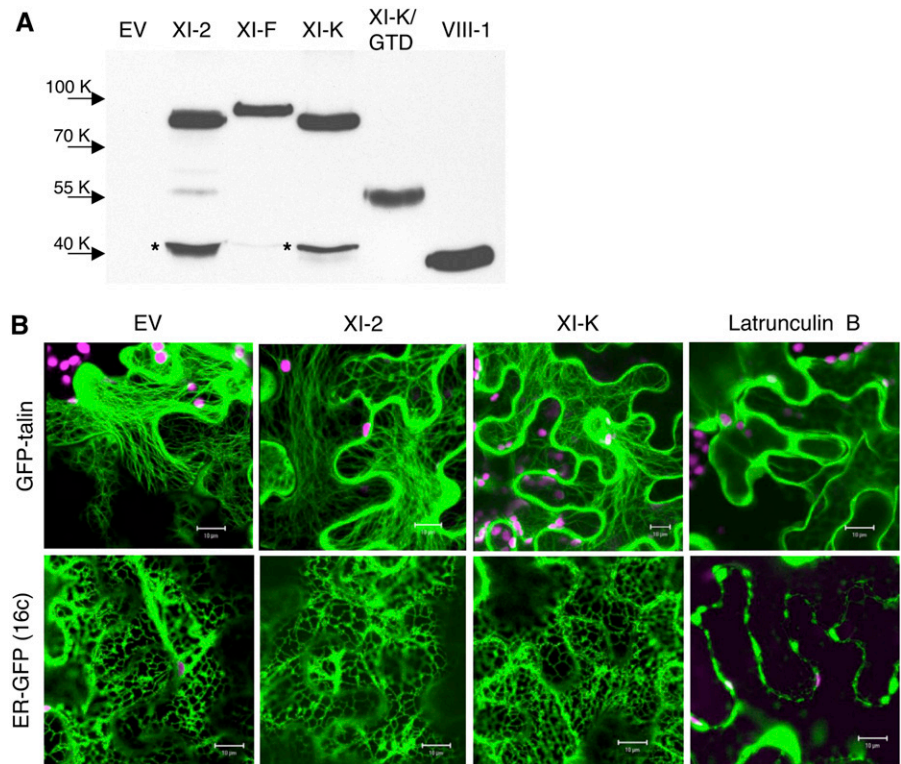
To investigate the contribution of the tail subdomains to the observed inhibition of Golgi trafficking, GTDs that possess no IQ and coiled-coil regions were expressed (Fig. 1B). Once again, overexpression of the XI-K GTD interfered with Golgi trafficking and reduced the mean velocity by approximately 10-fold, whereas GTDs of the other five tested myosins had only moderate effects (Fig. 3, A and B; Supplemental Movies S1 and S3; data not shown). This result indicated that the XI-K tail-mediated suppression of Golgi trafficking did not require IQ or coiled-coil motifs and was likely dependent on interference with cargo binding rather than tail-mediated inhibition of the motor domain given that the latter tail activity was shown to require the coiled-coil region (Li et al., 2006).

To determine the statistical significance of the observed differences in organelle velocity, a general linear model analysis followed by Scheffe's multiple comparison test was performed. This approach was chosen given the varying number of replicates between the treatments. The logarithmic transformation was done prior to the analysis to achieve homoscedasticity of the variances for each treatment. This transformation also reduced the positive skewness of the data and thus approached normality. In addition to the mean values, the medians were also determined to obtain a central measurement that is not significantly affected by the skewness (Supplemental Table S3). This analysis confirmed that the decrease in the Golgi velocity in the presence of the entire tails or GTDs of myosin XI-K was statistically significant (Supplemental Table S3; Scheffe's group D).

Analyses of the peroxisome motility in the same leaf cells revealed that overexpression of myosin XI-K tails reduced mean velocity of the peroxisomes 15-fold compared to that in the empty vector control (Fig. 3B) and virtually arrested trafficking of this organelle. The effects of myosin XI-2 and XI-F tails were significant but less dramatic; an approximately 3-fold reduction of the peroxisomes' mean velocity was observed (Fig. 3, A and B; Supplemental Movie S1; Supplemental Table S3).

The GFP reporter-tagged mitochondria were examined in separate experiments that revealed a drastic reduction of mitochondrial trafficking by myosin XI-K

Figure 2. A, Immunoblot analysis of myosin tail accumulation using HA-specific monoclonal antibody. Asterisks mark degradation products (Pashkova et al., 2006). B, Myosin tail overexpression does not affect actin microfilaments or the ER network in the leaf epidermal cells of *N. benthamiana*. Top row, Actin microfilaments are labeled by expression of GFP-talin. The actin cytoskeleton in the cells that express tails of myosin XI-2 or XI-K appears similar to control cells treated with the empty vector (EV), while treatment with the microfilament-disassembling drug latrunculin B results in disintegration of the actin cytoskeleton. Bottom row, The ER is labeled by ER-GFP in transgenic plant line 16c. The ER network whose structure is maintained by the actin microfilaments is intact in cells that express tails of myosin XI-2 or XI-K, but is collapsed in the presence of latrunculin B. B. Bars = 10 μ m.



tails. None of the other myosin XI or VIII tails had a significant effect on trafficking of this organelle (Fig. 4; Supplemental Movie S4). Taken together, these experiments suggested that, in leaf cells of *N. benthamiana*, myosin XI-K is required for trafficking of Golgi stacks, peroxisomes, and mitochondria, and, in addition, myosins XI-2 and XI-F functionally contribute to the trafficking of peroxisomes.

The analyses of individual organelle motility in control samples showed that the directions of translocation were apparently random with no dominant pattern for each of the three organelles (Figs. 2A and 3A, columns EV). The predominant translocation mode was saltatory rather than continuous. The velocity range was very broad, with some organelles being nearly immobile over the observation period and others moving large distances (Supplemental Movies S1 and S4), resulting in high SDs of the velocity values (Supplemental Table S2). Furthermore, we found that the mean velocities for the Golgi stacks and peroxisomes in the same set of leaf cells differed more than 2-fold under certain treatments (Fig. 3B; Supplemental Table S2). These results indicated that individual Golgi stacks, peroxisomes, and mitochondria move independent of each other rather than passively following the current of cytoplasmic streaming.

Transient RNAi of Myosin XI-K in *N. benthamiana* Reduces Organelle Trafficking

Systemic and local RNAi were used to confirm a major role of myosin XI-K in organelle motility by an

independent approach. The systemic RNAi assays were performed in GFP-transgenic plants to monitor the overall efficiency of RNAi by disappearance of green fluorescence (Fig. 5A). Because the Golgi stacks were tagged with the YFP reporter and the nucleotide sequence of the YFP gene differs from that of the GFP transgene, GFP silencing eliminated GFP fluorescence but did not compromise analyses of Golgi trafficking (Fig. 5B). The RNAi experiments with myosins XI-2, XI-F, and XI-K were performed by using the corresponding inverted repeat constructs whose efficiency was confirmed in separate experiments (Supplemental Fig. S2). The analyses of Golgi stacks and peroxisome trafficking showed that abrogation of myosin XI-K expression resulted in major reduction of the translocation distances (Fig. 5B; Supplemental Movie S5) and velocity (Fig. 5D; Supplemental Table S2) for both organelles. In contrast, RNAi of the myosin XI-2 or XI-F had only a moderate effect on the trafficking of these organelles.

Mitochondrial trafficking was analyzed using local RNAi whose efficiency and specificity was confirmed by quantitative PCR analyses (Supplemental Fig. S2). As shown in Figure 5, C and D, and Supplemental Movie S6, motility of mitochondria was slightly reduced by RNAi of myosins XI-2 and XI-F but virtually abolished by RNAi of myosin XI-K. Statistical analyses confirmed that the results for myosin XI-K RNAi were significantly different from those for two other myosins (Supplemental Table S3). Collectively, the RNAi experiments supported the principal role of myosin XI-K in the rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in the leaf cells of *N. benthamiana*.

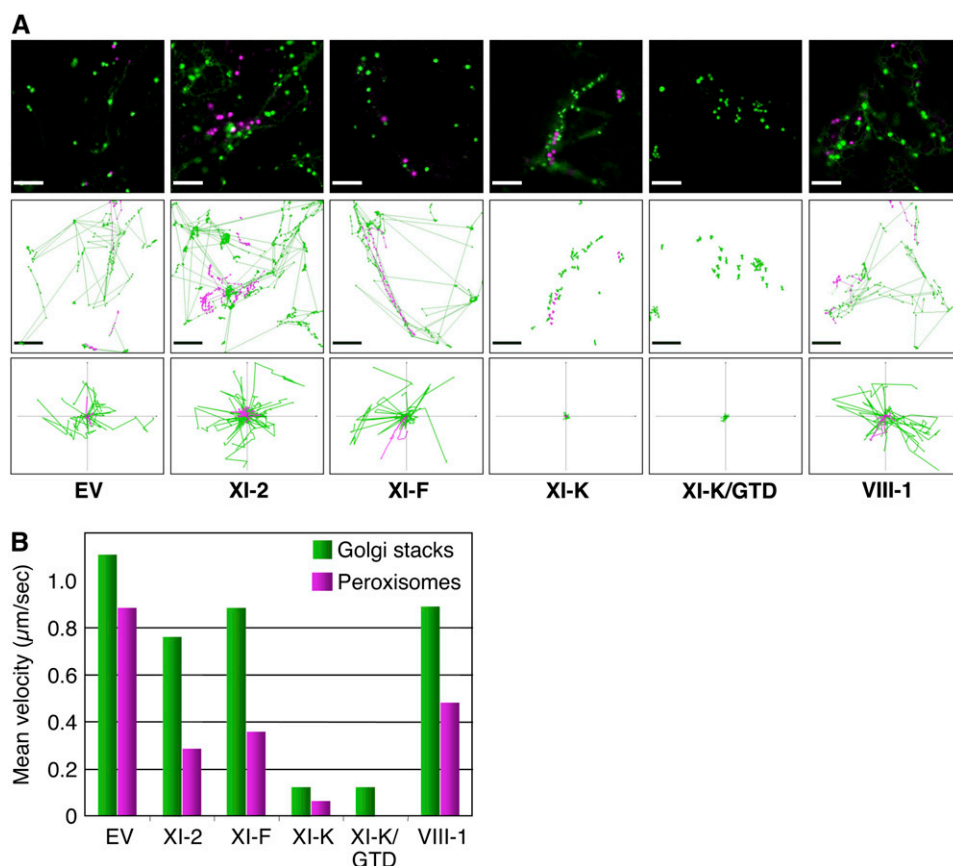


Figure 3. Effects of myosin tail overexpression on trafficking of Golgi stacks (green) and peroxisomes (magenta) in the leaf epidermal cells of *N. benthamiana*. **A**, Each column corresponds to expression of the myosin tail variant marked at the bottom; EV, empty vector control. Top row, Representative confocal images. Middle row, Dots and connecting lines show paths of individual organelles recorded in a time series. Bottom row, Movement of individual organelles plotted relative to a common origin. Bars = 7 μm . **B**, Mean velocity of the Golgi stacks and peroxisomes in the presence of overexpressed myosin tails as indicated below the diagram.

Actin-Dependent, Light-Induced Movements of Chloroplasts Do Not Require Myosin XI-K

The leading role of myosin XI-K in trafficking of the three distinct organelles prompted the question if this same myosin is required for the light-induced relocation of chloroplasts. Drug treatments were used to confirm that the movements of *N. benthamiana* chloroplasts in response to changing light depended on the integrity of actin microfilaments rather than microtubules (data not shown). The potential role of each of the six available *N. benthamiana* myosins in both bright-light avoidance movement and blue-light attraction movement of chloroplasts was assayed using overexpression of the cognate myosin tails. Surprisingly, none of the tested myosin tails had any detectable effect on chloroplast relocation under either bright or blue light illumination (Fig. 6). This result indicated that the light-regulated chloroplast translocations require distinct myosin motors that are yet to be identified and also confirmed the specificity of myosin XI-K function in trafficking of Golgi stacks, mitochondria, and peroxisomes.

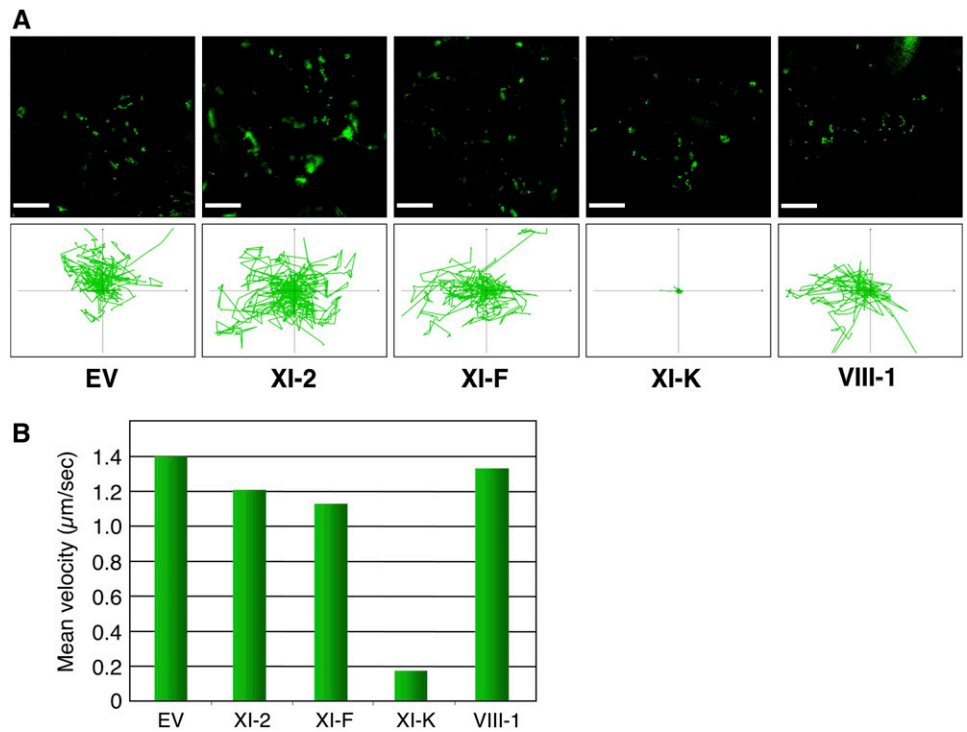
DISCUSSION

Membrane-bounded organelles are a defining feature of the eukaryotic cell. Among those, mitochondria are universally present in eukaryotes (Embley and

Martin, 2006); their arrival at the evolutionary scene via endosymbiosis, undoubtedly, was a major event in eukaryotic evolution and might have triggered the entire process of eukaryogenesis (Martin and Koonin, 2006). The endomembrane secretion pathway that includes the ER, the Golgi complex, and specialized derivatives, such as peroxisomes, is another pan-eukaryotic feature (Lee et al., 2004; Scheckman, 2005; Gabaldon et al., 2006; Hanton et al., 2006). The morphology, distribution, and inheritance of the membrane-bounded organelles are dependent on the cytoskeleton that provides the infrastructure of the eukaryotic cell and carries molecular motors that are responsible for the physical translocation of the organelles (Vale, 2003; Pruyne et al., 2004). Comparative functional genomics using diverse models, such as yeast, vertebrates, and plants, is gradually progressing toward a unifying picture of the eukaryotic cell dynamics defined by the interactions between the organelles and the cytoskeleton.

The actomyosin system is, arguably, the most ancient of the cytoskeletal motility systems of the eukaryotes (Vale, 2003). The well-established correlation of the myosin superfamily evolution with the origins of the major eukaryotic lineages suggests that myosin is not only one of the "founder" eukaryotic proteins but also plays an important role in defining the lifestyle of the eukaryotic organism (Richards and Cavalier-Smith, 2005; Foth et al., 2006). The early diversification of myosins in the common ancestor of Magnoliophyta

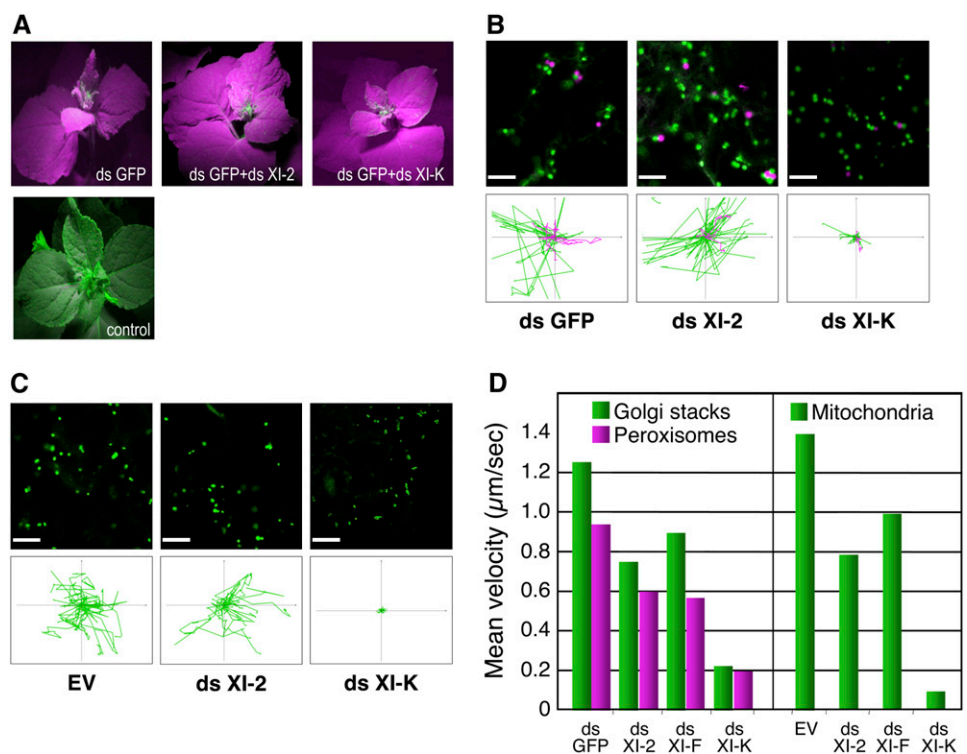
Figure 4. Effects of myosin tail overexpression on trafficking of mitochondria (green) in the leaf epidermal cells of *N. benthamiana*. A, Images and trafficking patterns of mitochondria. Bars = 14 μm . B, Mean velocity of the mitochondria in the presence of overexpressed myosin tails. The designations are the same as in Figure 3.



described here is compatible with this notion. Plants exhibit the ultimate case of the actomyosin-driven, namely, incessant organelle motility that involves reshaping and repositioning of the nucleus and vacuole, photo-induced chloroplast movements, and the fastest known trafficking of mitochondria, Golgi

stacks, peroxisomes, and other endomembrane organelles (Wada et al., 2003; Shimmen and Yokota, 2004; Wada and Suetsugu, 2004). So far, however, none of the numerous plant myosins of the classes XI and VIII was assigned a specific function in the organelle motility. The experiments described here and in an accompa-

Figure 5. Transient RNAi of myosin XI-K mRNA in *N. benthamiana* inhibits trafficking of Golgi stacks, peroxisomes, and mitochondria. A, Images of the GFP-transgenic, systemically silenced plants used in the experiments shown in B, taken under UV (top row). The nonsilenced, empty vector control is shown at the bottom. B, Images and trafficking patterns of Golgi stacks (green) and peroxisomes (magenta) in plants systemically silenced for GFP only (ds GFP), GFP and myosin XI-2 (ds XI-2), or GFP and myosin XI-K (ds XI-K). Bars = 7 μm . C, Images and trafficking patterns of mitochondria (green) in plants locally silenced for myosin XI-2 (dsXI-2) or XI-K (dsXI-K). EV, Empty vector control. Bars = 14 μm . D, Mean velocity of the organelles in transiently silenced plants.



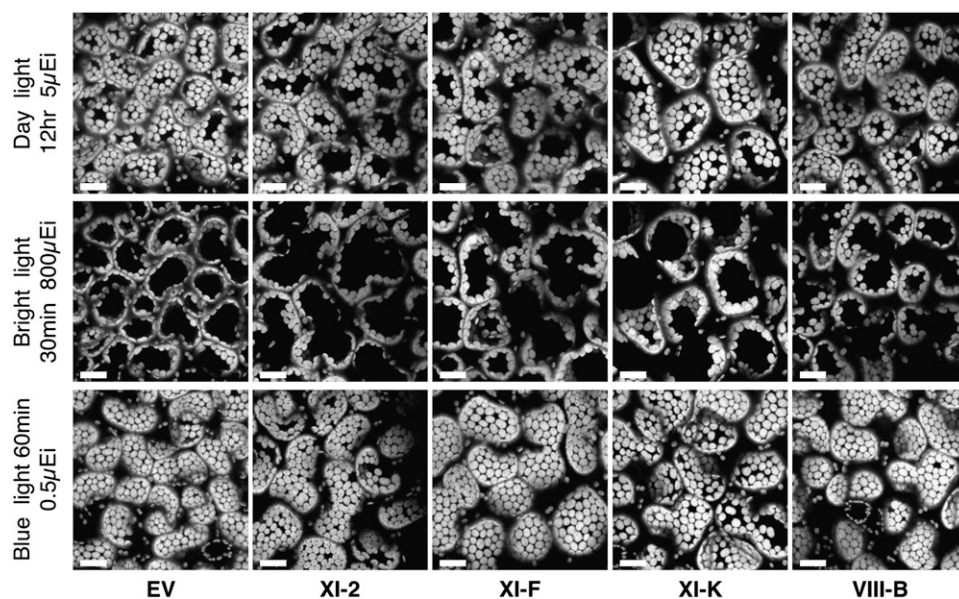


Figure 6. Bright light-avoidance and blue light-attraction movements of chloroplasts in *N. benthamiana* leaf epidermal cells are unaffected by ectopic expression of the myosin tails. Irradiation time and light intensity is shown at the left of each row; the identities of the overexpressed myosin tails are shown below each column. Bars = 20 μm .

nying article (Peremyslov et al., 2008) start to fill this gaping information void by identification of the myosin species that is responsible for the organelle trafficking.

We used *N. benthamiana* (family *Solanaceae*) to identify the myosin that is largely responsible for the continuous trafficking of the Golgi stacks, mitochondria, and peroxisomes, and to investigate translocation patterns of these organelles. First, we isolated six class XI and class VIII myosins from *N. benthamiana* and expressed the cognate cargo-binding tails in plants that possessed fluorophore-tagged organelles. This dominant negative approach was used to interfere with the myosins' ability to bind and transport their respective cargoes. Surprisingly, it was found that the tails of one and the same myosin, XI-K, dramatically reduced processive movement and mean velocity of each of the three studied organelles. Substantially less pronounced, albeit statistically significant effects of other myosin tails can be explained by the incomplete specificity of the approach (i.e. potential cross reactivity of myosins in the dominant-negative test) or by the certain contributions of these myosins to organelle trafficking. The latter possibility appears very likely in the case of the *N. benthamiana* myosins XI-2 and XI-F that might play a supportive role in peroxisome trafficking as suggested by substantial reduction in peroxisome velocity upon expression of the corresponding GTDs.

To validate the results obtained using overexpression of the truncated myosins, we employed transient RNAi to knock down *N. benthamiana* myosins. As expected, RNAi for myosin XI-K, but not those for any of the other myosins, inhibited substantially trafficking of Golgi stacks, mitochondria, and peroxisomes. These results support the principal role of myosin XI-K in the organelle translocation in this plant species.

None of the tested tails of myosins XI or VIII interfered with the light-induced chloroplast move-

ments. Because it has been shown previously (Paves and Truve, 2007) and confirmed here that chloroplast repositioning depends on the actomyosin motility system, it is likely to involve a dedicated myosin motor(s) that remains to be identified.

A comparison of the results described here with the results of analogous experiments in *Arabidopsis* that are reported in the accompanying article (Peremyslov et al., 2008) suggests that orthologous myosins could have overlapping but not identical functional profiles in the two plants. Indeed, myosin XI-K plays a prominent role in the trafficking of Golgi stacks, peroxisomes, and mitochondria in both species. However, it appears that *Arabidopsis* myosin XI-2 is (almost) as significant as myosin XI-K for movement of these three organelles in *Arabidopsis*, but not in *N. benthamiana*, where the myosin XI-2 roles appear to be subordinate (Peremyslov et al., 2008). These findings are compatible with the reports that *Arabidopsis* myosin XI-2 associates with peroxisomes (Hashimoto et al., 2005; Li and Nebenfuhr, 2007; Reisen and Hanson, 2007).

The functions of plant myosins XI-K and XI-2 in the transport of Golgi stacks and peroxisomes is reminiscent of yeast class V myosin Myo2p that is responsible for mechanical translocation and segregation of these organelles during cell division (Pruyne et al., 2004). This same myosin is also required for the proper inheritance of yeast mitochondria, although its role might be indirect (Boldogh and Pon, 2006). Given the close phylogenetic relations between class V and class XI myosins, this overlap in functional profiles suggests that functions of myosin XI-K and XI-2 in organelle trafficking might descend directly from the original function of the ancient myosin V. The remarkable proliferation of the class XI myosins in plants (Fig. 1A) compared with only one to three myosins V in fungi or metazoa suggests functional diversification of the class XI myosins and, more generally, a greater significance

of actomyosin-dependent organelle trafficking in plants. Furthermore, the exceptionally fast processive movement of organelles mediated by plant class XI myosins (Tominaga et al., 2003) underlies the extreme avidity of intracellular dynamics that is one of the most prominent features of plant cells. Given the diversity of plant myosins, the main result of this work, namely, that the same myosin is responsible for trafficking of three major organelle types, comes as a surprise. The lack of assigned functions for the remaining plant myosins indicates that major mechanistic features of plant cell dynamics remain to be elucidated.

The concept of cytoplasmic streaming defined as a coordinated flow of the cytosol that carries smaller organelles has become a staple of biology textbooks (Buchanan et al., 2000; Taiz and Ziegler, 2006). Cytoplasmic streaming is well documented in filamentous algae and is traditionally applied to land plants as well (Shimmen and Yokota, 1994, 2004; Shimmen, 2007). However, analyses of organelle trafficking presented here and in previous studies (Nebenfuhr et al., 1999; Logan and Leaver, 2000; Nebenfuhr and Staehelin, 2001) seem to warrant a revision of this concept. Indeed, our simultaneous analyses of the trafficking patterns and velocities for the thousands of Golgi stacks and peroxisomes in the *N. benthamiana* cells revealed features that appear incompatible with indiscriminate streaming. First, we observed no coordination or preferential direction in organelle movement patterns. Second, these movements were saltatory rather than continuous. Third, the mean velocities of Golgi stacks and peroxisomes determined in the same cells were significantly different at least in some experiments (Supplemental Table S2). Independent analyses of mitochondrial trafficking also showed saltatory, multidirectional movements indicative of independent trafficking of the individual organelles.

Our results do not rule out more uniform patterns of organelle movement in certain parts of the cell or certain cell types. Indeed, the flow of the peroxisomes in a predominant direction was seen in parts of elongated cells such as the cells of vascular epidermis or root hairs (Peremyslov et al., 2008). From a mechanistic point of view, even this directional flow is compatible with actively moving organelles perhaps being followed by the cytosol, but not vice versa. Therefore, we propose that active, myosin-dependent, multidirectional movement of individual organelles along microfilaments is the dominant mechanism of organelle trafficking in higher plants. In the specific cases of linear organization of the actin cytoskeleton that is seen in elongated cells (Hepler et al., 2001; Smith and Oppenheimer, 2005), this active organelle movement has an appearance of uniform cytoplasmic streaming.

Although rapid organelle trafficking appears to be characteristic of all green plants, its exact functional significance remains unclear. In our experiments, immobilized mitochondria and peroxisomes were frequently seen in close association with the chloroplasts (not shown). Observations of frequent encounters be-

tween these organelles and chloroplasts also have been reported by others (Logan and Leaver, 2000; Mano et al., 2002). These findings suggest that organelle hopping might be important for redistribution of energy and metabolites within the cell and that this hopping might be ordered rather than random. It also appears reasonable to assume that rapid trafficking of Golgi stacks facilitates delivery of the secreted macromolecules from the ER to their peripheral destinations (Boevink et al., 1998; Nebenfuhr et al., 1999). Therefore, it seems that the cytoplasmic stirring mediated by myosin-powered organelle hopping emerges as a conceptual replacement of cytoplasmic streaming in higher plants. Further characterization of the class XI myosins initiated in this and accompanying work will undoubtedly provide important clues as to the mechanisms, functional significance, and regulation of the plant cell dynamics.

MATERIALS AND METHODS

Isolation and Sequencing of Myosin cDNAs of *Nicotiana benthamiana* and Bioinformatics Analyses

A conserved region in myosin mRNA was amplified using degenerate primers as described (Bezanilla et al., 2003). Cloning of the complete myosin cDNAs was done using FirstChoice RLM-RACE kit (Ambion). The cDNAs encoding the complete tails and GTDs of each myosin were cloned into modified binary vector pCB302 (Prokhnovsky et al., 2005) downstream from the triple HA tag (primer sequences are available upon request). Phylogenetic analyses were performed using the MOLPHY program to build unrooted maximum-likelihood trees (Adachi et al., 2000) on the basis of a multiple alignment constructed using the MUSCLE program (Edgar, 2004). Poorly aligned regions were removed manually; the final alignment used for the phylogenetic reconstructions shown in Figure 1A included 580 positions. The MOLPHY program was also used to compute the RELL bootstrap probabilities (Adachi et al., 2000).

Ectopic Protein Expression

All binary expression vectors were transformed to *Agrobacterium tumefaciens* strain C58 GV2260, and the resulting bacteria were used for the *N. benthamiana* leaf infiltrations at 0.2 to 0.5 OD₆₀₀ (Prokhnovsky et al., 2005). The expression of the truncated myosin variants was assayed by immunoblotting using anti-HA monoclonal antibody (Roche) at approximately 18 h post-infiltration for each infiltrated area following organelle trafficking analysis (below). The fluorophore-tagged organelle markers were coexpressed with myosin variants by mixing corresponding bacterial strains of the same suspension densities to ensure similar protein expression levels in all cells. A Golgi-specific reporter was obtained by fusing A-2,6-sialyltransferase (Saint-Jore et al., 2002) with YFP as described (Prokhnovsky et al., 2005). A fusion between mCherry (Shaner et al., 2004) and pumpkin (*Cucurbita pepo*) hydroxypyruvate reductase (Mano et al., 2002) was used to tag the peroxisomes. The fusion of the *Nicotiana glauca* β -ATPase with GFP in a plasmid pBINmgfp5-atpase (Logan and Leaver, 2000) was used as a mitochondrion-specific reporter.

Organelle Trafficking

Confocal laser scanning microscopy was performed using a Zeiss LSM 510 META microscope fitted with the following configurations of excitation and emission filters, respectively: 488 nm and 508 nm for GFP, 513 nm and 527 nm for YFP, and 587 nm and 610 nm for mCherry. For time-lapse experiments, the consecutive images were taken at 2-s intervals. Confocal movie clips (25 frames) were analyzed using the Velocity 3.7.0 Classification software (Improvision, Image Processing and Vision Company; <http://www.improvision.com/products/velocity/>) using the setting recommended in the software manual for all measurements. All organelles present in each clip were analyzed and

two-dimensional movement was recorded. The mean track velocity (micrometers per second) was calculated for a minimum of 10 movie clips; over 400 individual organelles were recorded using three to four leaves from different plants per each treatment (Supplemental Table S2). It should be emphasized that clips were obtained for dozens of individual cells picked at random to avoid any bias.

Transient RNAi

The inverted repeat constructs harboring *N. benthamiana* myosin sequences (nts 3,146–3,375 in the myosin XI-2 open reading frame, 3,143–3,370 for myosin XI-F, and 3,153–3,357 for myosin XI-K) were generated in pRTL2-based plasmid (Johansen and Carrington, 2001) with intron 1 from FAD2 separating the sense and antisense DNA (Stoutjesdijk et al., 2002). The silencing cassette was cloned into pCB302 and mobilized into *A. tumefaciens* C58 GV2260. In the local RNAi assays, the silencing strains were coinfiltrated with the marker strains, and the organelle movement was recorded 24 h later. For the systemic RNAi, each silencing construct was supplemented with inverted repeat derived from nts 1 to 172 of the ER-GFP transgene present in 16c line of the *N. benthamiana* plants (a gift from D.C. Baulcombe, University of Cambridge, UK). The bottom leaves of the 3-week-old 16c plants were agroinfiltrated three times with 1-week intervals. The organelles were visualized by the infiltration of the marker strains to upper silenced leaves 4 to 5 weeks later, and the movement was recorded after additional 24 h of propagation.

The quantitative PCR was done using Reverse-iT first-strand synthesis kit (ABgene), 500 µg of the leaf RNA with anchored oligo(dT), and Absolute QPCR SYBR Green mix (ABgene) in the Rotor-Gene 3000 machine (Corbett Life Sciences). The results were analyzed by the software provided by the manufacturer; primer sequences are available upon request.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ875135 (XI-2), DQ875136 (XI-F), DQ875137 (XI-K), DQ875138 (VIII-1), DQ875139 (VIII-2), and DQ875140 (VIII-B).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Multiple alignment of the motor domain of plant myosins which was used for phylogenetic tree reconstruction.

Supplemental Figure S2. RNAi of the myosins in *N. benthamiana*.

Supplemental Table S1. Classification of plant myosins.

Supplemental Table S2. Organelle movement data.

Supplemental Table S3. Statistical analysis of the organelle velocity (micrometers per second).

Supplemental Movie S1. Effects of the myosin tail overexpression on the trafficking of Golgi stacks and peroxisomes in the leaf epidermal cells of *N. benthamiana*.

Supplemental Movie S2. Effects of myosin VIII-2 and VIII-B tail overexpression on trafficking of Golgi stacks and peroxisomes in the leaf epidermal cells of *N. benthamiana*.

Supplemental Movie S3. Effects of myosin GTD overexpression on trafficking of the Golgi stacks in the leaf epidermal cells of *N. benthamiana*.

Supplemental Movie S4. Effects of myosin tail overexpression on trafficking of mitochondria in the leaf epidermal cells of *N. benthamiana*.

Supplemental Movie S5. Effects of systemic RNAi of the myosins XI-2 and XI-K on the trafficking of Golgi stacks and peroxisomes in the leaf epidermal cells of *N. benthamiana*.

Supplemental Movie S6. Effects of local RNAi of the myosins XI-2 and XI-K on the trafficking of mitochondria in the leaf epidermal cells of *N. benthamiana*.

ACKNOWLEDGMENTS

We are grateful to Jim Carrington, John Fowler, and Todd Mockler for useful discussions and critical reading of the manuscript, and to Amit Gal-On for kindly providing lab space to D.A. We thank David Baulcombe, Chris

Hawes, David Logan, Shoji Mano, and Roger Tsien for providing plasmids and transgenic plant lines. The authors acknowledge the Confocal Microscopy Facility of the Oregon State University Center for Genome Research and Biocomputing.

Received November 27, 2007; accepted December 24, 2007; published January 4, 2008.

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