

Chapter 2

Plant Myosins

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2.1 Introduction

Myosin is a molecular motor capable of producing motive force along actin filaments using the energy from ATP hydrolysis. A myosin molecule is generally composed of heavy and light chains. Most of the myosin heavy chains identified thus far have basically the N-terminal motor domain with ATP-hydrolysis and actin-binding sites, a neck domain with light chain binding sites, and a C-terminal tail region in which primary structures and sizes are diverse between myosin classes. The motor domain together with neck domain is often referred to as myosin head. The neck domain occupied by light chains works as a lever arm in the motor function. On the basis of sequence similarity of motor domain, myosins are divided into at least 24 classes [22]. Among them, three classes of myosins, VIII, XI and XIII, are plant specific [73, 113]. The *Arabidopsis thaliana* genome encodes seventeen myosins, four myosin VIII and thirteen myosin XI classes, while twelve myosin XI and two myosin VIII classes in *Oryza sativa* [37, 74]. Two genes encoding Myosin XIII, *Aclmyo1* and *Aclmyo2*, is found only in green alga *Acetabularia* [107]. Isoforms of myosin XI and myosin VIII of land plants are divided into 5 and 2 groups, respectively, by the phylogenetic analysis [3]. In *Arabidopsis*, some isoforms of myosin X (XI-A to XI-E and XI-J) and myosin VIII (ATM2 and myosin VIII B) are exclusively expressed in pollen, while other isoforms, MYA1 (XI-1), MYA2 (XI-2), XI-I, XI-K (myosin XI) and ATM1 (myosin VIII) in vegetative organs at relatively high levels [4]. The plant myosins are believed to be involved in various cellular functions in such as cytoplasmic streaming or nuclear, organelle and vesicle transport, cytokinesis, membrane trafficking, signal transduction and intercellular communication through the plasmodesmata. Recently, plant myosins are also shown to be utilized for the intra and intercellular movement of some kinds of plant viruses in the host plant cells [2, 28].

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The investigation of plant myosin had begun from attempting to elucidate the molecular mechanism of characean cytoplasmic streaming [82]. In 1970–1990, several proteins with ATPase activities had been isolated biochemically and reported as plant myosins (cited in [84]). However, none of these results have been reproduced. Myosin VIII designated as ATM1 [45] and myosin XI as MYA1 [44] were first identified and cloned from *Arabidopsis thaliana*. Because of similarity in overall domain structures, at the first discovery MYA1 was grouped with myosin V, phylogenetically most closed to plant myosins [22, 62]. At the same time, native myosins, the 170-kDa myosin with 170-kDa heavy chain from lily pollen [117], the *Chara* myosin with 225-kDa heavy chain from *Chara* internodal cell [33, 114] and the 175-kDa myosin with 175-kDa heavy chain from tobacco cultured cell BY-2 [122], were isolated, and then identified as myosin XI later [41, 63, 85, 99]. These native myosins showed the motile activity in vitro and an actin-activated ATPase activity. In addition, the recombinant head of *Chara* myosin XI [35, 36] and MYA1 [27] possessing the motile activities, were prepared. The biochemical and biophysical properties of native myosin XI and its recombinant forms were analyzed. On the other hand, the motile and the ATPase activity of myosin VIII and myosin XIII have not yet been reported, because neither the native molecules from plant materials nor functional recombinant forms were prepared. However, ATP- and actin-binding sites in the motor domain and light chain binding sites in the neck domain are also conserved in these myosins.

In the past two decades, antibodies against animal myosin, frequently myosin II, were used for identifying the myosin from plant materials [73]. However, neither the antibody against the lily pollen 170-kDa myosin XI nor *Chara* myosin XI cross-reacted with the myosin II, visa versa [114, 117]. The 175-kDa polypeptide recognized by an anti-myosin II antibody in germinating lily pollen was found to be clathrin [93], indicating necessity of reevaluation of plant myosins identified by the antibody against animal myosin.

The molecular biological approach with the mutant and expression analyses and the immunocytochemical studies with specific antibodies against individual myosin class revealed the intracellular function of plant myosins. Myosin XI is generally associated with organelles through its tail region and is involved in their transport and the cytoplasmic streaming in plant cells. The lily pollen 170-kDa myosin XI is co-localized with various organelles and vesicles [119] including mitochondria and vesicles [77] in pollen tubes. This myosin is also found in subapical regions of tubes [60]. One of the cargos transported by tobacco 175-kDa myosin XI [124] is endoplasmic reticulum (ER). Myosin VIII is localized in cell plate and plasmodesmata [7, 75], and its tail region is also targeted to the endosomes [25, 80]. Myosin XIII is found on organelles and vesicles, preferentially on chloroplasts at cell apex of growing conical tip in *Acetabularia* [107]. The organelles and vesicles transport by plant myosins is documented in detail in the Chapter.

In this Chapter, we focus mainly on the biochemical and biophysical properties and the role of heavy chain domains of myosin XI. Furthermore, we describe their relevance to physiological and cellular function, such as the cytoplasmic streaming or organelle and vesicle transport. We also discuss the mechanism of cytoplasmic

streaming in plant cells in order to further insight into its molecular basis and the role, based on a recently proposed model of cytoplasmic streaming in *Arabidopsis* [104]. The cytoplasmic streaming in *Arabidopsis* is indicated to participate in the organization of actin bundles and in the integration of cytoplasmic strands.

2.2 Domain Structures of Plant Myosin Heavy Chains

The molecular weight of plant myosin heavy chains deduced from their sequences ranges from 120 to 140-kDa for myosin VIII and from 150 to 270-kDa for myosin XI [113]. The heavy chains of plant myosins are composed of motor and neck domains and tail region. Figure 2.1 shows the schematic domain structures of heavy chains of plant myosins. In the N-terminus before the motor domain in myosin VIII, an extra peptide consisted of around 90 amino acids is present, although its function is unclear thus far [45, 73]. A PEST motif is included in this peptide [7, 106]. This motif is cleaved in vitro by a protease, calpain [65] and is thought to be responsible for degradation of the proteins including itself. This motif may be involved in the turnover of myosin VIII. In myosin XI heavy chain, the existence of putative Src homology 3 (SH3)-like subdomain is revealed in its N-terminus before the motor domain [37, 62, 109]. The function of this subdomain is also unclear at present.

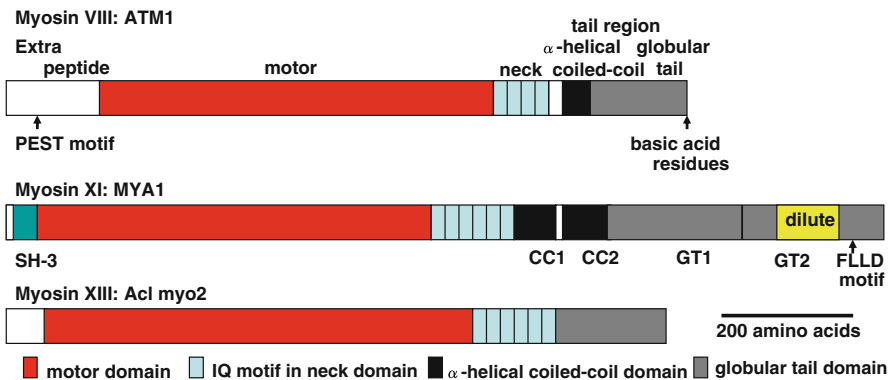


Fig. 2.1 Schematic domain structures of plant myosins, myosin VIII (ATM1), myosin XI (MYA1) and myosin XIII (Aclmyo2) (adopted from [62]). Before the motor domain, N-terminus extra peptides exist in plant myosins. In this peptide region of ATM1 or MYA1, PEST motif [7] or SH-3 like subdomain [37, 109] is present, respectively. In extreme C-terminus of ATM1, a group of basic amino acid residues are present [45]. The α -helical coiled-coil domain in MYA1 is divided into two segments, CC1 and CC2 [56]. Between them, a non-helical loop consisting of 10–15 amino acids, probably braking the α -helical coiled-coil structure, is inserted [56, 99]. The globular tail domain in MYA1 is composed of two subdomains, GT1 and GT2 [55]. In GT2 subdomain, a dilute domain, signature element of this myosin and myosin V [57], is present. FLLD motif is putative AtRabC2a binding site [30]. In myosin XIII, α -helical coiled-coil domain lacks

This domain identified in animal myosin classes is considered to interact with other proteins possessing the SH3 binding domain or with motor domain itself [62]. In *Dictyostelium* myosin II, this subdomain is indicated to communicate with the functional regions in the motor domain [23]. In neck domain of myosin VIII and myosin XI heavy chains, 3–4 and 4–6 tandem repeats of IQ motifs, respectively, responsible for binding of calmodulin (CaM) or its related proteins as light chains, are present. The tail region of myosin VIII and myosin XI is further divided into two domains, α -helical coiled-coil domain in its N-terminus and globular tail domain in the C-terminus. Within the C-terminus in the globular tail domain of myosin XI, a dilute domain designated from a mutant “dilute” mouse that encodes the heavy chain of myosin Va, is contained and is signature element of this myosin and myosin V [57]. At the very C-terminus in myosin VIII, a putative phosphorylation site by protein kinase A and C [7] and a group of basic residues [45] are present. Although plant myosins were classified based on sequence comparison of motor domains, the tail regions between myosin isoforms belonging in each class are also conserved to some extents, like non-plant myosins [49]. Two myosin XIII heavy chains, Aclmyo1 and Aclmyo2, are also consisted of motor and neck domain with 4 or 7 IQ motifs and tail region [107]. But, two unique features are found in the tail region. First the α -helical coiled-coil domain is lacking in both myosin XIII, and second the length of Aclmyo1 tail region is so short, only 18 amino acids are contained, whereas Aclmyo2 has long tail composed of 178 amino acids.

2.3 The Motor Domain

The motor domain is implicated in hydrolyzing ATP, interacting with actin filaments, and then generating the motive force along actin filaments cooperatively with the neck domain. The ATP binding pocket containing phosphate binding loop (P-loop) and surface loops for interacting with the actin are conserved in plant myosins, despite of several divergences in each class plant myosin, also in isoforms of each myosin class [41, 63, 74, 109]. Recombinant motor domain of *Chara* myosin XI [35, 36] and MYA1 [27] fused to artificial lever arm, instead of their intrinsic neck domain, can translocate F-actins in vitro, revealing that the motor domain of myosin XI actually possesses and exerts the motile activity. The motile activity of myosin VIII and XIII has not yet been reported. Similarly to the head containing motor and neck domains of MYA2 [109], a head of myosin VIII isoform, ATM2, expressed in tobacco leaf cells decorates the actin filaments [80]. The expression of tail region of tobacco myosin VIII or ATM1 inhibits the targeting of virus protein to the plasmodesmata in tobacco cells [2] or the movement of certain endosomes [25], respectively, in a dominantly negative manner. These results likely indicate the motor activity in myosin VIII.

The motile activity of myosin is usually measured and evaluated by using the motility assay in vitro. In a simple assay, the fluorescently labeled F-actin with ATP is introduced onto the myosin-coated glass surface in a flow cell and is monitored

by high sensitive camera [84]. By this assay, native lily pollen 170-kDa myosin XI [117], tobacco 175-kDa myosin XI [122] and *Chara* myosin XI [33, 114] showed the translocation of the F-actin with a velocity comparable to that of cytoplasmic streaming or organelle transport observed in living pollen tubes, BY-2 cells and characean internodal cells, respectively. The *Chara* myosin XI can slide the F-actin at the velocity of 50–60 $\mu\text{m/s}$, the fastest molecular motor in the world. The sliding velocity of F-actin generated by the recombinant MYA1 head in vitro is estimated to be 3.2 $\mu\text{m/s}$, corresponding to the velocity of the organelle movements in *Arabidopsis* [27]. The F-actin prepared from skeletal muscle is usually utilized for monitoring and analyzing the motile activity in vitro owing to its easy large scale preparation. No significant difference was shown in the sliding velocity induced by the lily pollen 170-kDa myosin XI between plant F-actin prepared from BY-2 cells and animal one [34]. Furthermore, a subfragment-1 (S1) of skeletal muscle myosin II, the single myosin head fragment, translocated both plant and animal F-actin with similar velocity. These evidences demonstrate the involvement of myosin XI in the cytoplasmic streaming or organelle transport, and that the myosin XI in different plant species has an intrinsic motile activity to slide actin filaments with different velocity. In tobacco BY-2 cells, other myosin XI isoform, which is composed of 170-kDa heavy chain cross-reacted with an antibody against the lily pollen 170-kDa myosin XI heavy chains, translocates the F-actin with different velocity, 3–4 $\mu\text{m/s}$, from that of tobacco 175-kDa myosin XI, an average velocity of 9 $\mu\text{m/sec}$ [122, 124]. Incidentally, the lily pollen 170-kDa myosin XI translocates F-actin in vitro at an average velocity of 7 $\mu\text{m/s}$. These observations suggest that the myosin XI isoform in a given plant species also possesses the different motile activity. Which differences in myosin head or other domains contribute to the distinct sliding velocity between myosins XI, and in particular how *Chara* myosin XI is able to have such fast sliding velocity remain to be elucidate, and are now in progress [32, 113].

2.4 The Directional Determinant of Organelle Transport by the Polarity of Actin Filaments

The actin filament and its bundle work as a track for myosin motors. The filament is a polar polymer; one end is usually referred to as barbed end (+ end) and the other as pointed end (– end), because the myosin II head, S1 or HMM (heavy meromyosin, two headed myosin fragment of skeletal muscle myosin II), decorates it as arrowhead fashion. The polymerization rate of barbed end is faster than that of pointed end, when the monomer G-actin is added to a pre-exist actin filament. On the other hand, the depolymerization rate on the pointed end is higher than that on barbed end (see Chapter). The individual class of myosin moves unidirectionally along an actin filament, and the most of myosin classes thus far move toward the barbed end, with an exception of myosin VI moving toward the pointed end [110]. Similarly to myosin II and myosin V, the tobacco 175-kDa myosin XI is shown to move along an F-actin toward the barbed end, but not opposite direction [99].

The cytoplasm in the characean cell streams toward barbed ends of actin filament cables visualized by their decoration with HMM [42]. When organelles isolated from lily pollen are introduced on actin cables of *Nitella* internodal cell, they can move along actin cables in the same direction as that of the myosin II-coated beads or as that of endogenous cytoplasmic streaming in *Nitella* [46, 48]). These evidences support that the *Chara* myosin XI and the lily pollen 170-kDa myosin XI, maybe even other myosins XI, are also barbed end-directed motors, and imply that the direction and polarity of the cytoplasmic streaming or organelle and vesicle movement generated by myosin XI are determined by the polarity of actin filaments or bundles, but not myosin XI. Like in characean cell, the cytoplasmic streaming occurs toward barbed ends of actin bundles in a root hair cell of *Hydrocharis* [98] and in a pollen tube of *Haemanthus* [54]. In some tip growing cells, the cytoplasmic streaming shows a reverse fountain pattern. The streaming is directed to the tip in the periphery of tubes, while to the base in the transvacuolar strand penetrating vacuole in the center of tubes. In the periphery and the transvacuolar strand, arrowheads of HMM on actin filaments in the bundles point to base and tip, respectively. These evidences strongly support the above implication. In an individual actin bundle in pollen tube [54] and root hair cell [98] and in an actin cable in characean cells [68, 111], the polarity of actin filaments shows uniform polarity. An actin binding protein, villin, which is able to arrange the actin filaments into a bundle with uniform polarity [118, 123], is colocalized with the actin bundles or cables in such plant cells [98, 105, 120, 123]. Hence, villin is one of the actin-filament bundling factors determining the polarity of actin filaments in their bundles. The electron microscopic images showing that the actin bundles are frequently linked to cortical microtubules in the periphery of pollen tube [52] and root hair cell [98], in which the organization of actin bundles is disturbed by the depolymerization of microtubules [96]. It is speculated that the cortical microtubules play a role in the polar arrangement of actin bundles in the periphery of cells. On the other hand, the other mechanism as described below is proposed for those polar arrangements in the transvacuolar strand, in which the microtubules are absent.

2.5 The Neck Domain

The neck domain in plant myosin heavy chains has several IQ motifs, interacting sites with CaM or its related proteins even in the absence of Ca^{2+} . Therefore, CaM is expected to be associated with the heavy chains as the light chain in plant myosins. CaM was identified to be light chain in lily pollen 170-kDa myosin XI [121] and tobacco 175-kDa myosin XI [122]. However, it is not certain whether all IQ motifs are occupied with CaM. The detail sequence analysis of IQ motif in *Chara* myosin XI with Pfam protein families database [36] or CaM target database [32] provided a possibility that other factors, instead of CaM are targeted to IQ motifs in this myosin. In chicken brain Va [14, 19] or yeast myosin V, Myo2p [91], essential light chains of myosin II, LC17 and LC23 or Mlc1p respectively, bind to the

neck domain, in addition of CaM. It is possible that other protein(s), besides CaM, is also associated with IQ motifs as light chains in other myosin XI.

It is generally accepted that the neck domain occupied by light chains acts as a lever arm for power stroke and force transduction (lever arm model) [101]. The step size, the displacement generated by myosin per one ATP hydrolysis cycle, is determined in part by the length of lever arm and is expected to become larger when the length of lever arm is longer. Myosin V, in which the neck domain is composed of 6 IQ motifs, can move along an F-actin with 36 nm step size. This size was shown to decrease with reducing the lever arm length, the number of IQ motifs [79]. The sliding velocity is also expected to be proportional to the lever arm length [101]. *Chara* myosin XI motor domain fused to the artificial lever arms with various lengths, such as no neck domain with 3 nm length of lever arm, two α -actinin repeats with 14 nm length that also acts as lever arm in myosin motor, and the neck domain of mouse myosin V containing 6 IQ motifs with 24 nm length, can translocate F-actins in vitro, and their sliding velocity is also proportional to the length of lever arm in some extents [35, 36]; at velocity of around 24 $\mu\text{m/s}$ by the motor domain with longest lever arm, while 8 $\mu\text{m/s}$ with shortest one. The lever arm model also appears to be applied to the myosin head of myosin XI. Similarly to myosin V, the tobacco 175-kDa myosin XI having 6 IQ motifs in the neck domain can move along F-actin with 35 nm step size, which was revealed by an optical trap analysis [99]. The length of 35 nm matches the half-pitch of F-actin helix, about 36 nm, indicating that tobacco 175-kDa myosin XI moves more or less straight, neither spirally nor rotationally, along the longitudinal axes of the actin filament. On the other hand, the step size of *Chara* myosin XI is estimated to be 19 nm [43], shorter than expected value from 6 IQ motifs. This discrepancy may be due the dissociation of light chains from neck domain during isolation steps, or the step size may not simply and solely be determined by the length of neck domain in native *Chara* myosin XI.

2.6 α -Helical Coiled-Coil Domain in the Tail Region

From the presence of α -helical coiled-coil domain, myosin VIII and myosin XI heavy chains have been predicted to form a dimer [44, 45]. The electron microscopic studies support this prediction. The *Chara* myosin XI [115] and tobacco 175-kDa myosin XI molecule [99] have two heads connected by stalk or rod structure, and a tail with two small globular structures (Fig. 2.2a). There are two remarkably morphological differences among these myosins XI. In the head, the shape and size of *Chara* myosin XI is similar to that of myosin II, while the tobacco 175-kDa myosin XI to myosin V. Second difference is the length of stalk. The tobacco 175-kDa myosin has 25 nm stalk, consistent with whole length of α -helical coiled-coil domain, about 24 nm, deduced from its primary sequence (about 170 amino acids) of MAY1 heavy chain [56]. This domain in other higher plant myosins XI is also composed of similar number of amino acid residues. On the other hand, *Chara* myosin XI has a very long rod structure, about 50 nm [115], reflecting the long

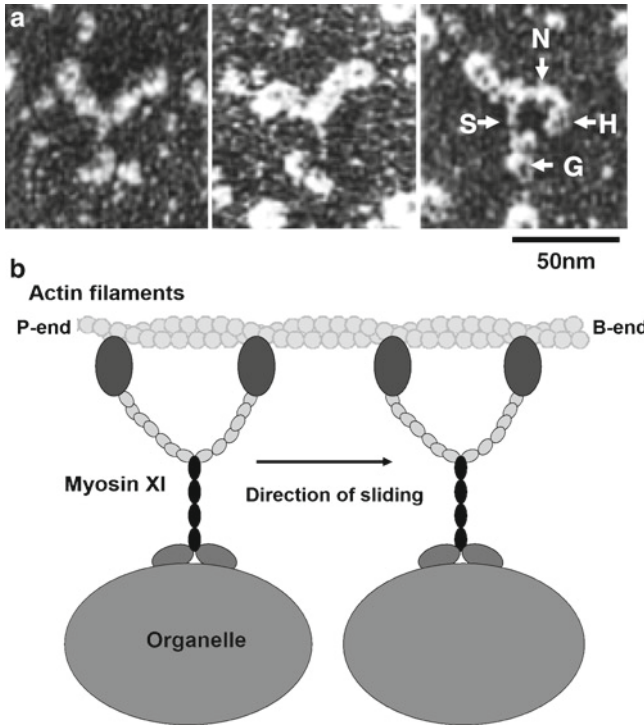


Fig. 2.2 The electron microscopic images of tobacco 175-kDa myosin XI (**a**) (adapted from [99]) and a schematic model showing the transport of organelles by myosin XI (**b**) (adapted from [85]). (**a**), The motor domain (arrow *H*), neck domain containing 6 IQ motifs (arrow *N*), a stalk (arrow *S*) or rod constructed from dimerized α -helical coiled-coil domains, and two small globular tail subdomains (arrow *G*), are visualized. (**b**) Myosin XI is associated with organelle through its globular tail domain. The organelles are translocated by the sliding of myosin head along actin filaments from their pointed end (P-end) toward the barbed end (B-end)

α -helical coiled-coil domain, in which around 750 amino acids are included [32, 67]. The first 191 residues exhibit relatively higher similarity with the sequence in the α -helical coiled-coil domain of MYA1 [32].

The α -helical coiled-coil domain in higher plant myosin XI is further divided into two segments, CC1 and CC2 [56]. Between these segments corresponding to the center of α -helical coiled-coil domain, there is a non-helical loop consisting of 10–15 amino acids, probably breaking the α -helical coiled-coil structure [56, 99]. Expressed α -helical coiled-coil domains of MAY1 in *Arabidopsis* leaf epidermis show the weak interaction between them, conceivably the dimerization [56]. The weak interaction between α -helical coiled-coil domains is dependent predominantly on the CC1 segment. This segment appears to be highly conserved in other higher plant myosins XI, predicting their similar properties in the dimerization. Interestingly, the interaction between α -helical coiled-coil domains is stabilized by the organelle targeting through the globular tail domain as described below.

Despite the absence of α -helical coile-coil domain in myosin XIII heavy chain [107], the situation in situ, monomeric or dimeric, is not known. While native myosin VI is monomeric, its dimerization via the tail domains is promoted by associating with adaptor/receptor proteins [71, 126] or phosphatidyl inositol 4,5 bisphosphate (PIP₂) [89] on the cargo. This may be true for myosin XIII.

2.7 Processive Movement of Higher Pant Myosin XI

The most important feature of motor proteins responsible for the transport of cargo is processivity; a single myosin motor moves along an actin filament for a long distance without detaching during the multiple mechanochemical cycles [11, 26, 100]. Hence, even small numbers of processive myosin is able to transport the cargo effectively along the actin filament for long distance. In animal cells, myosin V, but not all, and myosin VI have been known as the processive motors, which shows properties in their mechanochemical cycles different from those of non-processive myosins, for instance, the rate-limiting release of ADP and a high duty ratio, meaning that myosin heads spend the majority of their overall ATPase cycle strongly bound to actin filament. The linkage of two heads with the stalk is absolutely important for prosseicve movement, in which at least one of two heads remains bound to the actin filament at all times during the movement. Yeast myosin V, Myo4p, kinetically possesses pocessive properties, the high duty ratio, however this myosin moves non-processively because it is monomeric, single headed myosin (cited in [10]). The non-processive myosins, for example myosin II, are dissociated from actin filament after a single mechanochemical cycle. To exert the function of non-processive myosins, in some cases, multiple motor complex or cluster is formed and assembled, for a famous example, the thick filament of myosin II in the muscle. The kinetical analyses in ATPase cycle and a single myosin-coated bead assay using the optical trap reveal the tobacco 175-kDa myosin XI is a fastest processive motor able to slide along an F-actin at the velocity of 7 $\mu\text{m/s}$ with 35 nm step in the known motor proteins [99]. To explain how myosin V is able to move processivity, a hand-over-hand model was proposed [81, 100]. The one head called as trailing head and the other as the leading head exchange coordinately and alternatively the position along an actin filament like as “walking” on the filament, and one head remains bound to the actin filament when the other head detaches from it. The similar mechanism is reliably applicable for the motion of tobacco 175-kDa myosin XI [99]. The recombinant head of MYA1 shows the kinetic processivity [27], ADP release as rate-limiting step and the high duty ratio. The dimerization of MYA1 via α -helical coiled-coil domain as described above, likely confers the processivity to this myosin.

In the bead assay using the optical trap, a force generated by single tobacco 175-kDa myosin XI is estimated to be 0.5 pN [99]. Hydrodynamic analysis has proposed that the motive force of cytoplasmic streaming per unit interface was 0.3–0.4 pN/ μm^2 in characean cells [125]. If the similar situations, such as the viscosity of cytoplasm

described below, are also applied to tobacco BY-2 cells, a few molecules of this myosin is assumed to be sufficient for transport of a spherical organelle with 1 μm diameter [99]. ER is indicated to be one of cargos translocated by this myosin in BY-2 cells [124]. This organelles forms strands and reticular structures and streams dynamically and continuously in the cytoplasm. The processivity of tobacco 175-kDa myosin XI, also of other higher plant myosins XI, may make a possible to effective transport of ER and other organelles.

On the other hand, whether the *Chara* myosin XI is the processive motor is controversial and remains to be elucidated (supporting non-processive in [6], processive in [32, 92]). The bead assay coated with a single myosin molecule with the optical trap shows the non-processivity in *Chara* myosin XI [43]. Even if this myosin is a non-processive motor, a multiple and high density of myosin molecule on the cargos along an actin filament, comparable with thick filament of myosin II, may enable *Chara* myosin XI effectively to transport cargos [113]. This situation is hypothesized to be one mechanism for fast sliding velocity in this myosin [43].

2.8 Regulation of Higher Plant Myosin XI Through CaM Light Chain

CaM is well known to regulate the activity of its target proteins in a Ca^{2+} -dependent manner. Hence, the CaM light chains are expected to confer the Ca-sensitivity to the myosin XI and to regulate its motile activity. In the case of lily pollen 170-kDa myosin XI [121] and tobacco 175-kDa myosin XI [122], their motile activities in vitro are suppressed by Ca^{2+} at concentrations higher than 10^{-6} M. In lily pollen 170-kDa myosin XI, the relation of inhibitory effect of Ca^{2+} on its motile activity with the behavior of CaM light chain was analyzed in some details. Between 10^{-6} and 10^{-5} M Ca^{2+} , the suppression of motile activity of lily pollen 170-kDa myosin XI is reversible, when the Ca^{2+} concentrations is reduced. The CaM remains associated with the heavy chain in this range of Ca^{2+} concentrations, seeming that Ca^{2+} in this concentration range causes reversible inhibition by an allosteric interaction of the heavy chain and the CaM light chain. In contrast, at higher than 10^{-5} M, Ca^{2+} induces an irreversible inhibition of the motile activity, and the dissociation of the CaM from the lily pollen 170-kDa myosin XI heavy chain. The impaired activity is restored by lowering Ca^{2+} concentrations to some extents in the presence of exogenous CaM prepared from the lily pollen. According to the lever arm model, the mechanism of CaM-dissociation-induced inhibition is suggested to be caused by reduction of the structural integrity in the lever arm. The dissociation of CaM light chains from the heavy chain by the high concentrations of Ca^{2+} had been indicated in myosin V and also caused the inactivation of motile activity in this myosin [50]. In living plant cells, however, it is improbable that the myosin encounters Ca^{2+} concentrations higher than 10^{-5} M.

2.9 Regulation of Cytoplasmic Streaming or Organelle Transports in Pollen Tubes

The individual organelle and vesicle show the characteristic moving patterns in the reverse fountain streaming, and positioning in the growing pollen tubes (see the Chapter). A steep tip-focused Ca^{2+} gradient is generally formed in a growing pollen tube, and the concentrations of Ca^{2+} are shown to be higher than 10^{-6} M in the extremely apical region of the tube [31]. The relatively large organelles, amyloplast and vacuole, turn back to the grain near the basis of the tip. Although the mitochondria and ER move to and locate in a subapical domain of the tip, they do not invade into the apical region ([60, 69]; and see Chapter). When the Ca^{2+} -gradient is diminished, these organelles stream into the apex, indicating that the cytoplasmic streaming or organelle transport is suppressed by Ca^{2+} , in particular at the apex of growing pollen tube. The movement of isolated organelles from lily pollen along actin cables in *Nitella* internodal cells is inhibited reversibly by Ca^{2+} at concentrations between 10^{-6} and 10^{-5} M [46, 47], similarly to lily pollen 170-kDa myosin XI. These observations suggest that myosins in pollen tubes possess the Ca-sensitivity in their motile activities, acting as suppressive in the presence of Ca^{2+} higher than 10^{-6} M, and that the Ca-sensitivity in myosins is one of the physiological relevance for the Ca^{2+} regulation of cytoplasmic streaming. The dynamics and the organization of actin filaments in the apical and subapical regions are different from the shank of tubes and are modulated and regulated by Ca^{2+} through the actin binding proteins ([12, 13] and see Chapter). Hence, both “actin-linked” and “myosin-linked” Ca^{2+} regulation may work in concert for regulating the cytoplasmic streaming and organelle transport in pollen tubes.

2.10 Globular Tail Domain

From the similarity to myosin V, the globular tail domain of myosin XI has been deduced to be responsible for the cargo binding [44]. Based on the crystal structure of yeast myosin V, Myo2p, a predicted molecular architecture of MYA1 globular tail domain from its primary sequence is depicted and shows well assemble in that of globular tail in Myo2p [55]; subdomains, GT1 (globular tail 1) and GT2, are present and are associated tightly with each other in MYA1 globular tail domain. The interaction between two subdomains is confirmed in yeast two-hybrid screening and in *Arabidopsis* leaf cells. The live imaging approach, in which the tail region tagged with fluorescent protein is co-expressed with organelle and vesicular marker proteins in higher plant cells, provides the direct evidence that the tail region is actually targeted to the cargo. When the tail region containing α -helical coiled-coil and globular tail domains of several *Arabidopsis* myosin XI isoforms is expressed in the tobacco, onion and *Arabidopsis* cells, it is associated with Golgi and peroxisomes [55, 76]. In some cases, the movement of these organelles and mitochondria is

suppressed or modified dominant negatively by the expression of the tail region of several Myosin XI isoforms ([3, 70, 87] and see Chapter). Each subdomain, GT1 and GT2, of *Arabidopsis* several myosin XI isoforms, can be targeted independently with each other to same or distinct organelles [55]. For instances, GT2 of MYA1 or MYA2 is targeted to peroxisome, while GT1 of MYA1 or MYA2 to preferentially peroxisome and occasionally Golgi or unknown organelles, respectively. These observations extensively support that myosin XI is associated with the cargos through its globular tail domain (Fig. 2.2b), and that the globular tail domain possesses at least two organelle binding sites in GT1 and GT2.

When the construct containing α -helical coiled-coil and globular tail domains of myosin VIII, *Arabidopsis* ATM1 [25] or ATM2 [80], is expressed in *Arabidopsis* and tobacco cells, it is targeted to the plasmodesmata and endosomes. The localization of this myosin class in the plasmodesmata has been also revealed by the immunocytochemical studies using the specific antibodies against this myosin class [7, 75]. These expression analyses further indicate that myosin VIII is also involved in the membrane recycling in plant cells, and that its globular tail domain plays a role in targeting this myosin to the cargo or proper locate in plant cells. The basic residue group at very C-terminus in myosin VIII [45] is probably responsible for the direct binding of this myosin to acidic membrane lipid, like *Chara* myosin XI described below.

2.11 The Mechanism for Cargo Recognition by Higher Plant Myosin XI

Targeting organelles of *Arabidopsis* myosin XI tail region are not completely consistent with binding organelles of its subdomains. MYA1 tail region is targeting to organelles including Golgi, but not to peroxisome to which GT1 and GT2 of MYA1 are targeted. Other myosin XI isoforms showed similar behaviors to that of MYA1, suggesting the presence of a mechanism for selecting cargos of myosin XI in situ. Myosin V in animal and yeast is well known to interact with adaptor/receptor proteins in many cases through its globular tail domain for recruiting to the proper cargos [57]. Together with this evidence, a following model for explaining the selecting mechanism of myosin XI is predicted [55]. Subdomains, GT1 and GT2, take different conformations alternatively in myosin XI tail. Initially the tail domain binds to or interacts weakly with organelle-specific adaptor. This interaction selects one of these conformations, and then the tail domain is locked effectively on the organelle.

Small GTPase Rab proteins of *Arabidopsis*, AtRabD1 and AtRabC2a, are found to bind weakly to the MYA2 globular tail domain in a GTP-dependent manner in vitro [30]. The Rab proteins interchange between the active GTP-bound and inactive GDP-bound forms, which adopt different conformations each other [1]. The GTP-bound form of Rab protein interacts with effector proteins and performs the function. In animal cell and yeast, Rab proteins are one of adaptor/receptor proteins for myosin V targeting to proper cargos and can interact directly or indirectly via

other proteins with myosin V globular tail domain. The fluorescent-protein-tagged AtRabC2a expressed in *Arabidopsis* leaf cells is co-localized with the peroxisome, one of MAY2 targeting organelles ([29], and see Chapter). Hence, the AtRabC2a is indicated to be an adaptor/receptor for targeting MYA2 to the peroxisome, and its weak interaction with MYA2 tail domain might be compatible with and support the model described above. But, whether or not the weak interaction of MAY2 with AtRabC2a occurs in situ is obscure.

After the weak interaction of globular tail domain with organelle-specific adaptor, such as AtRabC2a, and subsequent selection of one conformation, this domain should be locked effectively on the organelle surface. At present, this mechanism is not confirmed. In many cases of myosin V, other proteins, in addition to Rab proteins, act as adaptor/receptor for connecting this myosin to its targeting cargo [57]. For example, in animal melanocyte, Rab27a localizes to the membrane surface of melanosome and recruits the melanophilin acting as adaptor and binding to myosin V globular tail. Consequently, myosin V is connected to melanosome. Hence, other proteins are conceivable to be implicated in the locking mechanism for higher plant myosin XI. Another possibility cannot be ruled out that the direct binding of the globular tail to membrane lipids is involved in locking the myosin tightly on the surface of organelle membrane, as follows. The globular tail of *Chara* myosin XI is associated directly with membrane lipids probably through its basic amino acid clusters [67]. The clusters are also conserved in higher plant myosin XI [56, 57]. Globular tail of myosin VI interacts with both adaptor/receptor proteins and PIP₂ and is targeted to early endocytic vesicles through this interaction [89].

Both N- and C-terminuses in the MYA2 globular tail domain is necessary for the interaction with AtRabC2a [30]. Similar situation has been shown in the interaction of mammalian myosin Vb globular tail domain with Rab11 family proteins, such as Rab11a, Rab11b and Rab25 [53]. The two interaction sites in the myosin Vb globular tail with these Rab proteins is indicated as follows; one site is LEKNE motif locating in its N-terminus, and the other is LLLD motif in its C-terminus. A similar sequence to the LEKNE motif is not found in the N-terminal region of MYA2 globular tail domain [30]. However, FLLD motif corresponding to LLLD motif is present within 55 amino acids in the C-terminus not only in MYA2 globular tail domain, but also in other *Arabidopsis* myosin XI isoforms and tobacco 175-kDa myosin XI, with exceptions of *Arabidopsis* myosin XI-G and XI-J. It is tempting to deduce that the interaction of other myosin XI isoforms with AtRab proteins through this conserved motif is one of the selecting mechanism for targeting individual myosin XI isoform to proper and certain cargos or sites for exerting its function in situ.

MYA2 also interacts in vitro with the other AtRab protein, AtRabD1, whose targeting organelles or vesicles have not been identified [30]. The subdomain, GT1 and GT2, of MYA2 globular tail, can bind to unidentified organelles and the peroxisome, respectively [55]. An immunocytochemical study also shows the association of MYA2 with other organelles and vesicles that are not peroxisome [29]. It is plausible that the AtRabD1 recruits this myosin XI to these

organelles and vesicles. The identification of organelles associated with this AtRab protein will help elucidating of other function of MYA2 besides of peroxisome transport.

The tail region of myosin VIII ATM1 is targeted to some endosomes marked and decorated with GFP-tagged Ara6 [25], which is a plant Rab5 ortholog and regulates the endocytosis in *Arabidopsis* [102, 103]. Although a direct interaction of ATM1 with Ara6 in vivo or in vitro has not been examined, it is attractive to consider that myosin VIII is also associated with the Ara6 on endosomes.

2.12 Functional Inter-Domain Communication Between α -Helical Coiled-Coil and Globular Tail Domains

As described above, the globular tail domain of the several myosin XI isoforms without the α -helical coiled-coil domain, could not be associated with organelles, while the tail regions including both domains is targeted to organelles [55, 76]. Hence, the α -helical coiled-coil domain is important to adopt a favorable configuration in globular tail domain for binding to organelles, since the α -helical coiled-coil domain alone could not be associated with organelles [55]. Intriguingly, the targeting globular tail domains to organelles is found to stabilize the dimerization between α -helical coiled-coil domains in two tail regions, suggesting the existence of an inter-dependent relationship between dimerization in α -helical coiled-coil domain and organelle binding in globular tail domain within MYA1 [56]. According to this scenario, there is an equilibrium between monomeric and unstable dimeric forms of MYA1, and only targeting to organelles could be arranged into the stable and functional dimer possessing the processivity. However, the isolated tobacco 175-kDa myosin XI molecule is dimer as described above. Although further study is need, it is postulated that dimeric myosin XI is stable and not disassembled easily into monomer once the dimer is formed.

As described above, the dimer formation in myosin VI is induced through its binding to adapter/receptor proteins or PIP₂ on cargos. In this case, two heavy chains of this myosin are connected in their tails, unlike myosin XI. In animal myosin V molecule, dynamic intramolecular conformational change is induced and responsible for the self-regulation of its activity [100]. In the presence of micromolar Ca²⁺, the myosin V has an extended conformation (active state), whereas it forms a folded shape in the absence of Ca²⁺ (inactive state), in which the globular tail domain is folded back toward and interacts with head region. By Ca²⁺ binding to CaM in the neck region, the globular tail domain dissociates from head region, and the myosin Va activities are restored. An adaptor/receptor proteins for myosin Va, melanophilin, which is associated with Rab27a and recruits this myosin to melanosome, is found to activate the actin-activated ATPase of myosin Va in the Ca²⁺ free condition [58]. This finding suggests that binding to cargo induces the activation of myosin V by unfolding inactive folded states.

The folded and inactive conformation in the absence of Ca^{2+} has not been reported in myosin XI thus far.

2.13 Direct Binding of *Chara* Myosin XI to Phospholipid Vesicles

On the contrary to higher plant myosin XI, the *Chara* myosin XI [115] and its recombinant globular tail domain [67] are shown to bind directly to vesicles prepared from acidic phospholipids, such as phosphatidylserine and PIP_2 but not from neutral phospholipids. When an arginine cluster present in the globular tail is substituted to non-charged amino acids for reducing its positive charge, the binding affinity of globular tail domain decreases drastically, indicating the electrostatic interactions of acid phospholipids with the globular tail domain. The direct binding of myosin tail to acid phospholipids has been well known in animal myosin I [15]. The binding of *Chara* myosin XI to lipid is supposed to be tight and to overcome the shearing force generated during the sliding movement of this myosin heads along actin filament cables [67].

2.14 Cytoplasmic Streaming in Characean Cells

Cytoplasmic streaming is a coordinated and directed bulk flow of cytoplasm carrying smaller organelles and vesicles in plant cells [82]. Its molecular and regulatory mechanism has been extensively investigated by using characean cells, in which the cytoplasm rotates in the same direction stably with so fast velocity at several ten $\mu\text{m}/\text{sec}$. The investigation of plant myosin had begun from attempting to elucidate the molecular mechanism of characean cytoplasmic streaming. The hydrodynamic analysis had proposed that only movement of long curling filamentous [125] or membrane network organelles expanding into the whole cytoplasm [66] could generate the motive force for the cytoplasmic streaming from actin cable to the viscous cytoplasm in characean cells, about 0.7–2 poise compared with 0.01 poise in a water. These organelles have been considered to be ER [38]. Although unambiguous demonstration is lacking, binding of *Chara* myosin XI to ER has been suggested in *Characeae* [67, 116]. Kamitsubo described that transparent structure is running along the actin cables (personal communication to Shimmen in 1978). It was reported that ER-like structure is associated with actin cables [38]. An immunocytochemical study using antibodies against *Chara* myosin XI shows the localization of this myosin along actin cables as vesicular-like dots [63, 116]. ER strands labeled with fluorescent probes rapidly streams in subcortical cytoplasm [21]. From these observations, an association of *Chara* myosin XI with ER has been suggested.

2.15 The Regulation of Cytoplasmic Streaming in the Characean Cell

In characean cells, the cytoplasmic streaming stops transiently. The necessity of extracellular Ca^{2+} had been indicated in this phenomenon [8]. Electrophysiological studies indicated the involvement of Ca^{2+} channel in generation of action potentials (cited in [86]). The generation of action potential induces the elevation of intracellular Ca^{2+} concentrations up to micromolar, and concomitantly the cytoplasmic streaming ceases transiently [112]. The membrane permeabilized cells, in which the cytoplasmic streaming is restored by the addition of ATP, the streaming is reversibly inhibited by micromolar Ca^{2+} [94]. However, this inhibitory mechanism is remained to be elucidated. Sliding of beads coated with skeletal muscle myosin II along actin cables is completely insensitive to Ca^{2+} , suggesting that characean actin cables are not equipped with Ca-sensitizing mechanism [83]. Therefore, myosin-linked Ca-regulation was suggested for the cytoplasmic streaming in *Characeae*. However, the actin activated ATPase and motile activities of isolated *Chara* myosin XI are affected by neither Ca^{2+} nor Ca^{2+} /CaM [5, 33, 114]. Hence, it is reasonable that Ca^{2+} does not affect directly *Chara* myosin XI activity (indirect myosin-linked Ca^{2+} -regulation). A phosphorylation-dephosphorylation has been suggested to be involved in the regulation of cytoplasmic streaming in characean cells [95]. Vesicle movement on the actin filaments peeling off from the surface of chloroplasts is observed in squeezed endoplasm from *Chara* cells in the presence of ATP, and is inhibited by the addition of phosphatase inhibitor, okadaic acid. On the other hand, it is activated by protein kinase inhibitor, staurosporine [64]. The sliding of F-actin on the glass surface coated with *Chara* myosin XI is similarly inhibited and activated by the treatment with the drugs. The motile activity is also diminished by the treatment with protein kinase C in the extract. The Ca^{2+} -dependent protein kinase, which also phosphorylates the substrate for protein kinase C, has been found to locate on the actin cables and on small organelle- or vesicle-like structures attached to the cables in characean cells [61], thereby making it possible to phosphorylate easily and specifically only *Chara* myosin XI just working on the cytoplasmic streaming. These evidences suggest two mechanisms of phosphorylation leading to dysfunction of *Chara* myosin XI in situ for suppressing the cytoplasmic streaming by Ca^{2+} . First is the inactivation of motile activity in *Chara* myosin XI by the phosphorylation. In animal, the motile and ATPase activities of several classes of myosins, such as ameba myosin I [15] and smooth muscle and non-muscle myosin II [16, 17], are regulated through the phosphorylation of head region or the regulatory light chain, respectively. The phosphorylation occurs in TEDS rule site residing between ATP- and actin-binding sites in motor domain in ameba myosin I and activates its activity. It is well known that the phosphorylation of regulatory light chain in the smooth muscle myosin and non-muscle II activates the ATPase activity and promotes thick filament assembly from the bent and folded monomeric molecule. Conversely, the phosphorylation also exerts the negative effect on the activities of myosin II, depending on the phosphorylation

site(s) in regulatory light chain. Which domain(s) or subunit(s) in *Chara* myosin XI molecule is phosphorylated and affects its motile activity is not clarified. Second is the dissociation of *Chara* myosin XI from the surface of cargos, such as ER. The consensus sequences of the phosphorylation site by the Ca^{2+} -dependent protein kinase and the Ca^{2+} /CaM-dependent protein kinase II are present near the arginine clusters in *Chara* myosin XI globular tail [67]. If this site is phosphorylated, the positive charge in the cluster is expected to be reduced, and the dissociation of myosin XI from organelles is anticipated. Similarly, myosin V tail has been reported to be dissociated from organelles by its phosphorylation [40]. It is naturally possible that both two inhibitory systems are exerted simultaneously.

2.16 Cytoplasmic Streaming in Higher Plant Cells

The streaming of Green fluorescent proteins expressed in BY-2 cells and diffused in their cytoplasm is found to be dependent on organelle movement [20], suggested that the cytoplasm is dragged by moving organelles. Recently, a reverse genetic approach using *Arabidopsis* mutant strain of myosin XI shows that the streaming ER is a force generating for the cytoplasmic streaming in *Arabidopsis* [104], similarly to characean cell. In *Arabidopsis*, strands of ER actively stream along the long axis of the cell, and the cytoplasmic streaming shows the similar velocity distribution to that of ER streaming. The streaming of ER is impaired, and the morphology and organization of ER is altered in mutants of myosin XI, especially myosin XI-K, indicating this myosin XI isoform primary contributor to ER streaming, which is implicated intimately in the cytoplasmic streaming. An inhibitor of myosin activities, BDM (2,3-butanedione monoxime) that can suppress the motile activity in vitro of lily 170-kDa myosin XI [97] and *Chara* myosin XI [24], also completely suppresses such ER dynamics. These observations further indicate that the sliding movement of myosin XI is involved in maintaining ER morphology. Interestingly, the organization and orientation of actin bundles in cytoplasm is also disturbed concomitantly, and transvacuolar strands disappeared in myosin XI mutant cells. For explaining these co-relations between myosin XI, ER streaming and actin-bundle organization, a following model is proposed. A portion of ER slides along disorderly and randomly oriented actin bundles through ER-associated myosin XI. The following ER streaming arranges the bundles into uniform polarity because of the unidirectional sliding of myosin XI, and forms thicker actin bundles due to aligning the adjacent bundles around ER through myosin XI. Reiteration of these interconnected processes induces a formation of longitudinally and unidirectionally oriented thick actin bundles, which provide tracks for the extensive streaming of ER strands and the structural integrity to transvacuolar strands. According to this model, dynamic interactions between ER streaming by myosin XI and actin bundles define the architecture and movement patterns of ER strands, the polarity and the orientation of actin bundles in cytoplasmic and transvacuolar strands.

However, how and where actin bundles are fixed or anchored within *Arabidopsis* cells are remained obscure. In the characean cells, actin cables are fixed on the immobile chloroplasts, thereby the organelles, maybe ER, could move relative to actin cables. Although chloroplasts are fixed to the gel ectoplasm in *Characeae*, they are translocated in response to various external stimuli in most higher plants [108]. In such plants, for example *Vallisneria* [18], *Arabidopsis* [39] and spinach [51], actin bundles are associated with and surround chloroplasts in circular or basket-like array, which is altered and reorganized depending on the chloroplast movement under the light conditions [18, 51, 78]. Hence, it is improbable to speculate that these actin bundles are involved in translocation of other organelles. One candidate organelle that tethers actin bundles is considered to be the cortical ER. ER network is continues between cortical ER, cytoplasmic ER and outer nuclear envelope [88, 90], and cytoplasmic ER is streaming through the sliding of myosin XI along actin bundles [104, 124]. On the other hand, the cortical ER does stream rarely as a whole, although it takes rapidly shape change and remodeling motions [88]. Actin bundles frequently locate close to or under the cortical ER network associated closely to cell membrane in higher plant cells [9]. This association of ER and cell membrane is relatively tight, overcoming to the centrifugal force [72]. Electron microscopic studies demonstrate that actin bundles attach to the cortical ER network along their several points and appear to be enwrapped with ER membrane [59]. Hence, it is likely that some portions of actin bundles are anchored to cortical region of cells by the cortical ER network.

2.17 Conclusion

Plant specific, three classes of myosins, myosin VIII, myosin XI and myosin XIII are identified and have similar domain structures, with an exception of lacking α -helical coiled-coil domain in myosin XIII. In myosin XI, the head region containing motor and neck domains works in generating motive force and binding to light chain CaM, the α -helical coiled-coil domain in dimerizing two heavy chains, and the globular domains in binding to cargos. In the case of myosin VIII and myosin XIII, the mechanochemical properties in motor head, composition of light chains in neck domain and binding mechanism to the cargo have not yet been shown thus far. Higher plant myosin XI can move processively with 35 nm step size along the actin filament, a favorable property effective in transporting its cargos even by small numbers of myosin molecules, and its motile activity is regulated by CaM light chain. This step size enables the myosin XI to move straight along the actin filament, and the straight motion with its cargos is expected to attenuate the viscous drag of cytosol comparing with the spiral motion. Although it is not defined whether *Chara* myosin XI is a processive motor thus far, multiple and high density of myosin molecule on organelle membrane surface close to actin cables may confer this myosin to transport organelles efficiently at fast velocity. One of these organelles may be ER. The streaming of ER causes the dragging force for cytoplasmic streaming. In *Arabidopsis*, the streaming of ER generated by myosin XI is

implicated not only in the cytoplasmic streaming, but also in sustaining the ER morphology and organizing actin bundles. Hence, it is possible that the orientation and polarity of actin bundles is arranged by sliding and moving of myosin XI associated with ER. The myosin XI via its tail region can bind directly membrane phospholipid of cargo or indirectly to the cargo through the adaptor/receptor proteins such as AtRab proteins. The tail region in myosin VIII is also responsible for the binding and targeting to the cargos, bacterial protein and endosome, and to the proper site, plasmodesmata. Further analyzing the organelle recognition mechanism should provide an insight into understanding the function of individual myosin XI or myosin VIII isoform, for example, total 17 isoforms in *Arabidopsis*, and the regulation of organelle and vesicle transport. With regard to myosin VIII, understanding mechanochemical properties in its motile activity will help further elucidating of its function in situ.

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