Molecular Characterization and Subcellular Localization of Arabidopsis Class VIII Myosin, ATMI

Received for publication, October 9, 2013, and in revised form, March 12, 2014. Published, JBC Papers in Press, March 17, 2014, DOI 10.1074/jbc.M113.521716

Takeshi Haraguchi‡1, Motoki Tominaga§1, Rie Matsumoto‡, Kei Sato¶, Akihiko Nakano§1, Keiichi Yamamoto‡, and Kohji Ito‡2

From the ‡1Department of Biology, Graduate School of Science, Chiba University, Inage-ku, Chiba 263-8522, the §1Live Cell Molecular Imaging Research Team, RIKEN Center for Advanced Photonics, Wako, Saitama 351-0198, the ¶1Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, and the §1Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

Background: Molecular properties of class VIII myosin are not characterized.
Results: Arabidopsis class VIII myosin, ATMI, has low enzymatic activity and high affinity for actin and is primarily localized at the cell cortex.
Conclusion: Our data suggest that ATMI functions as a tension sensor/generator.

Significance: This is the first report of enzymatic and motile properties of class VIII myosin.

Land plants possess myosin classes VIII and XI. Although some information is available on the molecular properties of class XI myosins, class VIII myosins are not characterized. Here, we report the first analysis of the enzymatic properties of class VIII myosin. The motor domain of Arabidopsis class VIII myosin, ATMI (ATM1-MD), and the motor domain plus one IQ motif (ATM1-1IQ) were expressed in a baculovirus system and characterized. ATM1-MD and ATM1-1IQ had low actin-activated ATPase activity (Vmax = 4 s−1), although their affinities for actin were high (Kact = 4 μM). The actin-sliding velocities of ATM1-MD and ATM1-1IQ were 0.02 and 0.089 μm/s, respectively, from which the value for full-length ATMI is calculated to be ~0.2 μm/s. The results of actin co-sedimentation assay showed that the duty ratio of ATMI was ~90%. ADP dissociation from the actin-ATMI complex (acto-ATMI) was extremely slow, which accounts for the low actin-sliding velocity, low actin-activated ATPase activity, and high duty ratio. The rate of ADP dissociation from acto-ATMI was markedly biphasic with fast and slow phase rates (5.1 and 0.41 s−1, respectively). Physiological concentrations of free Mg2+ modulated actin-sliding velocity and actin-activated ATPase activity by changing the rate of ADP dissociation from acto-ATMI. GFP-fused full-length ATMI expressed in Arabidopsis was localized to plasmodesmata, plastids, newly formed cell walls, and actin filaments at the cell cortex. Our results suggest that ATMI functions as a tension sensor/generator at the cell cortex and other structures in Arabidopsis.

Myosin is a motor protein that converts the chemical energy liberated by ATP hydrolysis into a directed movement on actin filaments. Phylogenetic analyses of myosin sequences reveal that there are at least 35 myosin classes (1), and their motor functions such as motility and ATP hydrolysis vary significantly. Only class VIII and XI myosins exist in higher plants. Arabidopsis thaliana possesses 4 and 13 genes encoding class VIII and XI myosins, respectively (2).

Studies on the intracellular functions of class XI myosins using immunolocalization, transfer DNA mutants, RNA interference, overexpression of dominant-negative myosins, and velocity-modified chimeric myosin XI indicated that they are responsible for organelle transport, organization of actin cable, and cell and plant growth (3–13). Molecular properties of several class XI myosins have also been characterized. The actin-sliding velocities and the actin-activated ATPase activities of class XI myosins are higher than those of other myosin classes (14–19). The dissociation rates of ADP from acto-class XI myosins are extremely fast and account for their high actin-sliding velocities (15, 19, 20).

In contrast to class XI myosins, considerably less is known about molecular properties and intracellular functions of class VIII myosins, although class VIII myosin, ATM1, was the first plant myosin to be identified and sequenced (21). The Arabidopsis genome encodes the following four class VIII myosins: ATM1, ATM2, VIIIA, and VIIIB (2). Immunolocalization experiments showed that ATM1 is localized to the plasmodesmata and new cell plates in the root of Arabidopsis (22). Analysis of the expression of GFP-fused tail domain of ATM1 suggests that endogenous ATM1 is localized to the plasmodesmata, endoplasmic reticulum, and plasma membranes (23). Although there is some evidence indicating the subcellular localization of class VIII myosins, definitive information regarding tissue-specific expression and subcellular localization using full-length myosin VIII expressed under the control of its native promoter have not been reported. In addition, molecular properties such as ATPase activity and actin-sliding velocity of class VIII myosin have not been characterized.

In this study, we expressed Arabidopsis class VIII myosin, ATM1, in a baculovirus system and uncovered its molecular
Enzymatic Properties of Class VIII Myosin

properties. Furthermore, its subcellular localization was determined by expressing GFP-fused full-length ATM1 in Arabidopsis under the control of its native promoter.

EXPERIMENTAL PROCEDURES

Constructs, Expression, and Preparation—Full-length cDNAs of Arabidopsis class VIII myosin, ATM1 (AT3G19960), and Arabidopsis calmodulin, CaM3 (AT3G56800), were provided by the RIKEN Bio Resource Center (24, 25). Baculovirus transfer vectors for ATM1-MD (pFastBac ATM1-MD) and ATM1-1IQ (pFastBac ATM1-1IQ) were generated using site-directed mutagenesis polymerase chain reactions (PCR) as follows. ATM1 cDNA was mutated to create an NcoI site at the 5′ end of nucleotide 1 of ATM1 and an AgeI site at the 3′ end of nucleotide 2526 of ATM1-MD or at nucleotide 2595 of ATM1-1IQ. PCR products were digested using NcoI and AgeI and ligated to the NcoI–AgeI fragment of pFastBac MD (19). These constructs (pFastBac ATM1-MD and pFastBac ATM1-1IQ) encode amino acid residues 1–842 of ATM1 or 1–865 of ATM1 for ATM1-MD and ATM1-1IQ, respectively, and also include a flexible linker (GGG), a Myc epitope sequence (EQKLISEEDL), and a His6 tag. ATM1-MD and ATM1-1IQ were expressed along with Arabidopsis calmodulin because the light chains of many unconventional myosins are calmodulin (26–38). Similar to many unconventional myosins (X, 29, 37), III, and VIIA chains of many unconventional myosins are calmodulin (26–38).

A baculovirus transfer vector for Arabidopsis CAM3 (pFastBac Arabidopsis calmodulin 3) was generated as follows. An Arabidopsis cDNA encoding CAM3 was mutated to create an XbaI site at the 5′ end and XhoI site at the 3′ end (after the translation termination codon) using site-directed mutagenesis PCR. PCR products were digested with XbaI and XhoI and ligated to the XbaI and XhoI fragment of pFastBac 1 (Invitrogen). This construct (pFastBac Arabidopsis calmodulin 3) encodes full-length Arabidopsis CAM3. This was expressed in insect cells and purified as described previously (19).

For the promoter-GUS (β-glucuronidase) assay, 3 kb of 5′-flanking sequences containing the first exon of ATM1 was PCR-amplified and subcloned into pENTR-D-TOPO (Invitrogen) and subsequently exchanged into the binary vector pGWB533 (40) using LR Clonase® (Invitrogen) according to the manufacturer’s instructions.

For transient expression in protoplasts, an ATM1 cDNA (AT3G19960) was amplified from total RNA purified from Arabidopsis seedlings (7-day-old) and subcloned into pENTR/D-TOPO (Invitrogen). The ATM1 sequence in the pENTR-D-TOPO cloning vector was exchanged into the binary vector pUGW0-sGFP using the LR reaction. For expression in Arabidopsis, full-length genomic ATM1 DNA, including the cDNA encoding sGFP upstream of the translational start codon, was generated by fluorescent tagging (41). The ATM1 fragment contains 3 kb of 5′- and 1 kb of 3′-flanking sequences. Amplified chimeric fragments were subcloned into the binary vector pGWB501 (40).

ATPase Activities and in Vitro Actin Gliding Assays—Steady-state ATPase activities were measured using a modified malachite green method (42). To avoid the possible inhibitory effect of ADP on binding of ATP to the active site, ATPase activities were measured when the molar ratio of ADP/ATP was less than 0.01.

The reaction mixtures for the assay of the basal Mg2+-ATPase activity contained 25 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 1 mM EGTA, 1 mM DTT, 2 mM ATP, 1 mg/ml BSA (Sigma, catalog no. A0281), and 4 μM ATM1-MD or ATM1-1IQ. The reaction mixtures for the assay of actin-activated Mg2+-ATPase activity contained 25 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 1 mM EGTA, 1 mM DTT, 2 mM ATP, 1 mg/ml BSA, 3–5 μM F-actin, and 0.005–0.03 μM ATM1-MD or ATM1-1IQ.

Actin-sliding velocities were measured using an anti-Myc antibody-based version of the in vitro actin-gliding assay as described (19). To avoid the possible inhibitory effect of ADP on actin-sliding velocities, an ATP regeneration system (0.4 mM phosphocreatine and 25 units/ml creatine phosphokinase) was included. To compare with other myosins (17, 19, 28, 29, 33, 34, 36, 43–48), the reactions of steady-state ATPase activities, in vitro actin-gliding assay, and kinetic measurements were conducted in the commonly used ionic strength (~50 mM) and temperature (25 °C) unless stated otherwise. The free [Mg2+] was 1.8 mM unless otherwise stated. The standard solution was as follows: 25 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA. In some experiments using either 0.17 or 0.46 mM of free [Mg2+] the ionic strengths were adjusted to 50 mM by changing the KCl concentration. The compositions of the assay mixtures were as follows: 1) 0.17 mM free [Mg2+]: 28 mM KCl, 1.7 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA; 2) 0.46 mM free [Mg2+]: 28 mM KCl, 2.3 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA. Free [Mg2+] and ionic strength were calculated using CALCON, which is based on Goldstein’s algorithm (49). ATPase activities and in vitro actin-gliding assays were done at 25 °C. For ATPase activities and in vitro actin-gliding assays of ATM1-1IQ, 1 μM Arabidopsis calmodulin was added in the assay buffer.

Co-sedimentation Assays—Co-sedimentation assays were performed as described (29), except that the solution was 170 mM in ionic strength (90 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 1 mM EGTA, 5 mM DTT, 10 mM phosphocreatine, and 50 units/ml creatine phosphokinase). In brief, 1 μM ATM1-MD was mixed with 0–96 μM actin and centrifuged at 280,000 × g for 20 min at 25 °C in the presence of ATP and the ATP generation system. Less than 12 μM actin, phallolidin–actin was used. The supernatant of each actin concentration was subjected to SDS-PAGE, and the fraction of ATM1-MD or pFastBac ATM1-1IQ was expressed in High FiveTM cells (Invitrogen) and kinetic measurements were conducted in the commonly used ionic strength (~50 mM) and temperature (25 °C) unless stated otherwise. The free [Mg2+] was 1.8 mM unless otherwise stated. The standard solution was as follows: 25 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA. In some experiments using either 0.17 or 0.46 mM of free [Mg2+] the ionic strengths were adjusted to 50 mM by changing the KCl concentration. The compositions of the assay mixtures were as follows: 1) 0.17 mM free [Mg2+]: 28 mM KCl, 1.7 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA; 2) 0.46 mM free [Mg2+]: 28 mM KCl, 2.3 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 10 mM DTT, 1 mM EGTA. Free [Mg2+] and ionic strength were calculated using CALCON, which is based on Goldstein’s algorithm (49). ATPase activities and in vitro actin-gliding assays were done at 25 °C. For ATPase activities and in vitro actin-gliding assays of ATM1-1IQ, 1 μM Arabidopsis calmodulin was added in the assay buffer.

Co-sedimentation Assays—Co-sedimentation assays were performed as described (29), except that the solution was 170 mM in ionic strength (90 mM KCl, 4 mM MgCl2, 25 mM Hepes-KOH (pH 7.4), 2 mM ATP, 1 mM EGTA, 5 mM DTT, 10 mM phosphocreatine, and 50 units/ml creatine phosphokinase). In brief, 1 μM ATM1-MD was mixed with 0–96 μM actin and centrifuged at 280,000 × g for 20 min at 25 °C in the presence of ATP and the ATP generation system. Less than 12 μM actin, phallolidin–actin was used. The supernatant of each actin concentration was subjected to SDS-PAGE, and the fraction of ATM1-MD bound to actin was determined by quantifying the amount of ATM1-MD in the supernatant using ImageJ software (National Institutes of Health). About 10% of ATM1 was precipitated in the absence of actin (0 μM actin). Thus, it is necessary to remove this artificial effect presumably caused by the aggregations of ATM1 to calculate the actual amount of acto-ATM1 precipitations. So, the amount of the pellet and the supernatant at 0 μM actin was set as zero and total amount of

12344 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 289 • NUMBER 18 • MAY 2, 2014
ATM1-MD, respectively. Therefore, the fraction unbound at each concentration of actin was calculated as follows: 100% \times (the amount of supernatant at each concentration of actin)/(the amount of supernatant at 0 \, \mu M \text{ actin}). The fraction bound was plotted as a function of actin concentration to determine the affinity of ATM1-MD for actin in the presence of ATP.

**Transient Kinetic Measurements**—All kinetic experiments were performed using an Applied Photophysics SX18MV stopped-flow spectrophotometer (dead time, 1.15 ms) as described (19). The solution used for the transient kinetic experiments was the same as that used for the ATPase activities and the in vitro actin-gliding assays unless stated otherwise.

**Transient Expression in Protoplasts of Arabidopsis**—A schematic diagram of native ATM1 deduced from its amino acid sequence is shown in Fig. 1A, Native ATM1. Native ATM1 includes motor and neck domains with four IQ motifs and coiled-coil and globular tail domains. It is likely that calmodulin or calmodulin-like light chains bind to each IQ motif and that native ATM1 forms a dimer through the interaction of its coiled-coil domain. We generated two recombinant ATM1 constructs called ATM1-MD and ATM1-1IQ (Fig. 1A, Recombinant ATM1). ATM1-MD contains a single motor domain, and ATM1-1IQ includes a motor domain and the first IQ motif. The expected lever arm lengths of ATM-1MD, ATM1-1IQ, and native ATM1 are 3, 7, and 19 nm, respectively. ATM1-MD and ATM1-1IQ were expressed using a baculovirus system. ATM1-1IQ was expressed along with Arabidopsis calmodulin. ATM-1MD and ATM1-1IQ were purified by co-precipitation with actin and nickel-affinity resin. For ATM1-1IQ purification, 1 \, \mu M Arabidopsis calmodulin was added to the purification buffer throughout the purification. Purified ATM1-1IQ contained calmodulin with stoichiometry 1:1 (Fig. 1B, with CaM). When external calmodulin was deleted before washing the Ni-NTA column, the stoichiometry decreased below 1:1 (Fig. 1B, wo CaM). These results suggest that calmodulin was weakly bound to the first IQ motif as a light chain, which was in equilibrium with the external calmodulin. It is also possible that the IQ motif might bind other light chains, not just calmodulin.

**RESULTS**

**Constructs**—A schematic diagram of native ATM1 deduced from its amino acid sequence is shown in Fig. 1A, Native ATM1. Native ATM1 includes motor and neck domains with four IQ motifs and coiled-coil and globular tail domains. It is likely that calmodulin or calmodulin-like light chains bind to each IQ motif and that native ATM1 forms a dimer through the interaction of its coiled-coil domain. We generated two recombinant ATM1 constructs called ATM1-MD and ATM1-1IQ (Fig. 1A, Recombinant ATM1). ATM1-MD contains a single motor domain, and ATM1-1IQ includes a motor domain and the first IQ motif. The expected lever arm lengths of ATM-1MD, ATM1-1IQ, and native ATM1 are 3, 7, and 19 nm, respectively. ATM1-MD and ATM1-1IQ were expressed using a baculovirus system. ATM1-1IQ was expressed along with Arabidopsis calmodulin. ATM-1MD and ATM1-1IQ were purified by co-precipitation with actin and nickel-affinity resin. For ATM1-1IQ purification, 1 \, \mu M Arabidopsis calmodulin was added to the purification buffer throughout the purification. Purified ATM1-1IQ contained calmodulin with stoichiometry 1:1 (Fig. 1B, with CaM). When external calmodulin was deleted before washing the Ni-NTA column, the stoichiometry decreased below 1:1 (Fig. 1B, wo CaM). These results suggest that calmodulin was weakly bound to the first IQ motif as a light chain, which was in equilibrium with the external calmodulin. It is also possible that the IQ motif might bind other light chains, not just calmodulin.

**Enzymatic Properties of Class VIII Myosin**

![Figure 1](image.png)

**Figure 1**. A, schematic diagrams showing native ATM1 and recombinant ATM1 constructs, ATM1-MD and ATM1-1IQ. The domain structure of native ATM1 was deduced from its amino acid sequence. B, SDS-PAGE of purified ATM1-MD and ATM1-1IQ. Purified ATM1-MD and ATM1-1IQ were analyzed using 4–20% SDS-PAGE and stained with Coomassie Brilliant Blue. For ATM1-1IQ (wo CaM), before washing the Ni-NTA-agarose column, external calmodulin was deleted. C, SDS-PAGE of purified ATM1-MD in the presence of EGTA or Ca^{2+}. Purified ATM1-1IQ was incubated with either 5 mM CaCl_{2} (+Ca^{2+}) or 5 mM EGTA (−Ca^{2+}) and then analyzed using 16% SDS-PAGE and stained with Coomassie Brilliant Blue. Low molecular mass band (arrow) shifts the mobility by Ca^{2+}, typical of calmodulin.

**Plant Transformation** —Plasmids were electroporated into Agrobacterium tumefaciens strain GV3101::pMP90 using a Gene Pulser (Bio-Rad). They were introduced into an Arabidopsis atm-1 knock-out line (SAIL_405_B08; AT3G19960) using the floral dipping method. T1 plants with resistance to hygromycin were selected. T2 generation plants were used for the promoter-GUS assay (50), and homozygous T3 plants were used for imaging full-length ATM1.

**Confocal Laser Scanning Microscopy** —GFP fluorescence in cells was detected using a spinning disk confocal laser scanning microscope (CSU10, Yokogawa, Kanazawa, Japan) equipped with a high resolution CCD camera (ORCA-AG, Hamamatsu Photonics, Hamamatsu, Japan). The images were processed using iVision Macintosh software (BioVision Technologies, Exton, PA).

**RESULTS**

**Constructs**—A schematic diagram of native ATM1 deduced from its amino acid sequence is shown in Fig. 1A, Native ATM1. Native ATM1 includes motor and neck domains with four IQ motifs and coiled-coil and globular tail domains. It is likely that calmodulin or calmodulin-like light chains bind to each IQ motif and that native ATM1 forms a dimer through the interaction of its coiled-coil domain. We generated two recombinant ATM1 constructs called ATM1-MD and ATM1-1IQ (Fig. 1A, Recombinant ATM1). ATM1-MD contains a single motor domain, and ATM1-1IQ includes a motor domain and the first IQ motif. The expected lever arm lengths of ATM-1MD, ATM1-1IQ, and native ATM1 are 3, 7, and 19 nm, respectively. ATM1-MD and ATM1-1IQ were expressed using a baculovirus system. ATM1-1IQ was expressed along with Arabidopsis calmodulin. ATM-1MD and ATM1-1IQ were purified by co-precipitation with actin and nickel-affinity resin. For ATM1-1IQ purification, 1 \, \mu M Arabidopsis calmodulin was added to the purification buffer throughout the purification. Purified ATM1-1IQ contained calmodulin with stoichiometry 1:1 (Fig. 1B, with CaM). When external calmodulin was deleted before washing the Ni-NTA column, the stoichiometry decreased below 1:1 (Fig. 1B, wo CaM). These results suggest that calmodulin was weakly bound to the first IQ motif as a light chain, which was in equilibrium with the external calmodulin. It is also possible that the IQ motif might bind other light chains, not just calmodulin.
TABLE 1
Steady-state ATPase activity of ATM1

| Constructs  | V_o | V_max | K_{actin} |
|-------------|-----|-------|-----------|
| ATM1-MD     | 0.014 ± 0.0005 | 4.1 ± 0.1 | 3.2 ± 0.4 |
| ATM1-IQ     | 0.012 ± 0.0007 | 4.7 ± 0.2 | 4.3 ± 0.3 |

*Mean ± S.D. of six independent measurements from two independent protein preparations.

Values are from the Michaelis-Menten equation (Fig. 2).

Figure 2. Actin-activated Mg^{2+}-ATPase activities of ATM1 as a function of free magnesium concentrations. Assays were performed in the presence of 0.17, 0.46, and 1.8 mM free [Mg^{2+}]. Values are averages of 3–9 assays from two to four independent preparations of ATM1-MD. The reactions were performed at 25 °C, and the data were fitted to the Michaelis-Menten equation. V_max values were lower by factors of 10–100 compared with those of plant class XI myosins (15, 17, 18) and are similar to those of animal unconventional myosins such as classes V (30), VI (51), and X (52). The difference may be explained by the differences in their lever arm length because the velocities of myosins are proportional to their lever arm length, if their motor domains are the same (18, 19, 44). Based on these results and lever arm length of native ATM1, we estimated that the velocity of native ATM1 is ~0.2 μm/s at 25 °C. This value is only 1% of those of plant-specific class XI myosins (15, 17, 18) and is similar to those of unconventional animal slow myosins such as classes V (30), VI (51), and X (52). These results suggest that ATM1 functions as a slow transporter and/or a tension generator.

Modulation of Actin-activated ATPase Activity and Actin-sliding Velocity by Free [Mg^{2+}].—Actin-activated ATPase activities and actin-sliding velocities of certain unconventional animal myosins that exhibit low actin-activated ATPase activities and slow actin velocities are regulated by free [Mg^{2+}] (53–56). Free [Mg^{2+}] in animal cells is not stable but varies temporally and spatially in the range 0.2–2 mM (57–70). Although studies about free [Mg^{2+}] in plant cells was limited, a similar concentration (0.2–2 mM) was estimated (71, 72). Therefore, we investigated whether actin-sliding velocity and actin-activated ATPase activities of ATM1 were regulated by free [Mg^{2+}] in this concentration range.

The relationship between velocities and free [Mg^{2+}] was the same for ATM1-MD and ATM1-IQ. When free [Mg^{2+}] increased, actin-sliding velocity decreased. Actin-sliding velocities in the presence of 0.46 and 0.17 mM free [Mg^{2+}] were higher by factors of 2 and 3, respectively, than in the presence of 1.8 mM (Fig. 3). Actin-activated ATPase activities also decreased when free [Mg^{2+}] increased. V_max of actin-activated ATPase activities of ATM1-MD at 0.46 and 0.17 mM [Mg^{2+}] were higher by factors of 2 and 3, respectively, than those in the presence of 1.8 mM [Mg^{2+}] (Fig. 2).

**ATP-induced Dissociation of Acto-ATM1.**—To unravel the molecular mechanism underlying slow actin-sliding velocity and the dependence of actin-activated ATPase activity and actin-sliding velocity on free [Mg^{2+}], we investigated the kinetic properties of ATM1. We used ATM1-MD for kinetic analyses because its steady-state ATPase activity and modulation of actin-sliding velocity by free [Mg^{2+}] were the same as those of ATM1-IQ (Table 1 and Fig. 3), although it is possible that there could still be differences between ATM1-MD and ATM1-IQ. Kinetic modeling and simulations of actomyosin were performed according to the simplified reaction mechanism as shown in Scheme 1, which has been used in kinetic studies of many myosins (28, 29, 34, 35, 56, 73–75).

We first investigated the molecular basis underlying slow actin-sliding velocity. In general, the actin-sliding velocities of myosins depend on the time when they are strongly bound to actin (t_i). t_i is primarily determined by the ADP dissociation rate from acto-myosin and the acto-myosin dissociation rate upon ATP binding at saturating ATP concentrations (76, 77). Therefore, we determined these rates.
The acto-myosin dissociation reaction upon ATP binding can be described as a two-step process (Scheme 2), and the rate constant is expressed as $K_1' = k_{-1}'/(1 + K_1'[ATP])$ (76).

The first step in Scheme 2 (where $A$ is actin, and $M$ is myosin) is the formation of a collision complex between acto-myosin and ATP, which are in rapid equilibrium. The second step is the dissociation of myosin-ATP from actin following the isomerization of myosin. To determine $K_1'$ and $k_{-1}'$, a pyrene-actin-ATM1-MD complex was dissociated by mixing with 10–3000 µM ATP using a stopped-flow apparatus, and the acto-ATM1-MD dissociation was monitored by the increase in pyrene fluorescence (Fig. 4) (78). The values of $K_1'$ and $k_{-1}'$ were obtained by fitting the data to $K_1'k_{-1}'[ATP]/(1 + K_1'[ATP])$. The values of $1/K_1'$ and $k_{-1}'$ were 450 ± 70 µM and 620 ± 40 s$^{-1}$, respectively (Fig. 4 and Table 4). The acto-ATM1-MD dissociation rate in the presence of a physiological concentration of ATP (2 mM) was 500 s$^{-1}$, which is extremely fast to explain the slow actin-sliding velocity of ATM1 (see “Discussion”).

**ADP Dissociation from Acto-ATM1**—The rate of ADP dissociation from acto-ATM1-MD was determined by measuring the decrease in the fluorescence intensity of mant-ADP (28, 32, 33, 75). Time course of fluorescence change followed a double exponential with fast $(k_{-1}^{\text{fast}}, 5.4 ± 0.4$ s$^{-1}$) and slow $(k_{-1}^{\text{slow}}, 0.35 ± 0.12$ s$^{-1}$) rate constants in the presence of 1.8 mM free [Mg$^{2+}$] (Fig. 5A and Tables 2 and 4). The relative amplitudes of the fast and slow phases were 82 ± 2 and 18 ± 2%, respectively. When pyrene-actin fluorescence was used to determine the ADP dissociation rate, similar double exponentials were observed (fast: $k_{-1}^{\text{fast}}, 4.7 ± 0.3$ s$^{-1}$, relative ampli-
Enzymatic Properties of Class VIII Myosin

TABLE 2

Free Mg$^{2+}$ dependence of ADP dissociation rates from acto-ATM1

| Free Mg$^{2+}$ | Fast $k_{5,\text{fast}}$ Amplitude | Slow $k_{5,\text{slow}}$ Amplitude | Signal |
|---------------|---------------------------------|---------------------------------|--------|
| mM            | Amplitude $x^{-1}$ | % | Amplitude $x^{-1}$ | % |
| 1.8           | 5.4 ± 0.4 82 ± 2 | 0.35 ± 0.12 18 ± 2 | mant-ADP |
| 4.7           | 0.3 ± 0.2 75 ± 1 | 0.46 ± 0.06 25 ± 1 | Pyrre-actin |
| 5.1           | 79 | 0.41 | 21 | Average $^a$ |
| 0.46          | 8.9 ± 0.7 88 ± 2 | 0.7 ± 0.1 12 ± 1 | mant-ADP |
| 7.0           | 0.5 ± 0.2 80 ± 2 | 0.54 ± 0.04 20 ± 2 | Pyrre-actin |
| 8.0           | 84 | 0.62 | 16 | Average $^a$ |
| 0.17          | 11 ± 0.2 88 ± 1 | 0.90 ± 0.2 12 ± 1 | mant-ADP |
| 10 ± 0.4       | 83 ± 1 | 0.50 ± 0.1 17 ± 1 | Pyrre-actin |
| 10.5          | 86 | 0.70 | 14 | Average |

Mean ± standard deviation of 3–4 independent measurements from two independent protein preparations.

$^a$ Relative amplitude of $k_{5,\text{fast}}$ and $k_{5,\text{slow}}$, respectively.

$^b$ Average of values from mant-ADP and pyrene-actin.

Mean ± standard deviation of 3–4 independent measurements from two independent protein preparations.

$^a$ Relative amplitude of $k_{5,\text{fast}}$ and $k_{5,\text{slow}}$, respectively.

$^b$ Average of values from mant-ADP and pyrene-actin.

The relative amplitudes of each fast and slow phase were almost constant for ADP binding to acto-ATM1 ($K_{5,\text{slow}}$) and is 0.12 (0.35/3.0 s$^{-1}$). The equilibrium dissociation constant for ADP binding to acto-ATM1 of the fast phase ($K_{5,\text{fast}}$) is calculated as $k_{5,\text{fast}}/k_{5,\text{slow}}$ and is 0.36 μM (5.4/1.6 μM$^{-1}$ s$^{-1}$). The overall equilibrium dissociation constant for ADP binding to acto-ATM1 ($K_{5,\text{overall}}$) is calculated as $K_{5,\text{fast}}(1 + K_{5,\text{slow}})$ (35, 55) and is 0.36 μM (Table 4). This value is similar to that of animal myosin Va (28) and is one of the lowest among all myosins, showing the high affinity of acto-ATM1 for ADP.

ADP Dissociation from ATM1—Kinetic parameters in the absence of actin were also measured. The rate of ADP dissociation from ATM1-MD was determined by measuring the decrease in fluorescence intensity of mant-ADP and followed by a double exponential with fast ($k_{5,\text{fast}}$) and slow ($k_{5,\text{slow}}$) rate constants. The rate of ADP dissociation from acto-ATM1-MD was also dependent on free [Mg$^{2+}$] concentrations (Table 3).

ADP Binding to ATM1—The rate of ADP binding to acto-ATM1-MD was biphasic with observed fast and slow rate constants (Fig. 5B), consistent with the two actomyosin-ADP states described above. The observed fast rate constants depended linearly on mant-ADP concentrations. The association rate constant of ADP with acto-ATM1-MD of the fast phase ($k_{5,\text{fast}}$) was $1.6 ± 0.11$ μM$^{-1}$ s$^{-1}$ (Fig. 5C, 5, fast and 5, slow). The relative amplitudes of each fast and slow phase were similar at every mant-ADP concentration, and the average values were $49 ± 5$ and $51 ± 5$% for fast and slow phases, respectively.

The equilibrium dissociation constant for ADP binding to acto-ATM1 of the slow phase ($K_{5,\text{slow}}$) is calculated as $k_{5,\text{slow}}/k_{5,\text{fast}}$ and is 0.12 (0.35/3.0 s$^{-1}$). The equilibrium dissociation constant for ADP binding to acto-ATM1-MD of the fast phase ($K_{5,\text{fast}}$) is calculated as $k_{5,\text{fast}}/k_{5,\text{slow}}$ and is 0.36 μM (5.4/1.6 μM$^{-1}$ s$^{-1}$). The overall equilibrium dissociation constant for ADP binding to acto-ATM1 ($K_{5,\text{overall}}$) is calculated as $K_{5,\text{fast}}(1 + K_{5,\text{slow}})(35, 55)$ and is 0.36 μM (Table 4). This value is similar to that of animal myosin Va (28) and is one of the lowest among all myosins, showing the high affinity of acto-ATM1 for ADP.

ADP Dissociation from ATM1—We subsequently investigated the dependence of ADP dissociation rate from acto-ATM1-MD ($k_{5}^*$) on free [Mg$^{2+}$] because the actin-sliding velocity and actin-activated ATPase activity of ATM1 were significantly affected by free [Mg$^{2+}$], and the rate-limiting step of these were ADP dissociations from acto-ATM1 as mentioned above. The value of $k_{5}^*$, particularly that of $k_{5,\text{fast}}$ depended strongly on free [Mg$^{2+}$] (Table 2). The values of $k_{5,\text{fast}}$ in the presence of 0.17 and 0.46 mM free [Mg$^{2+}$] were higher by factors of 2.7 and 1.6, respectively, than in the presence of 1.8 mM free [Mg$^{2+}$]. These results indicate that free [Mg$^{2+}$]-dependent modulation of the actin-sliding velocity and actin-activated ATPase activity of ATM1 is driven by free [Mg$^{2+}$]-dependent modulation of ADP dissociation from acto-ATM1.
the same at every mant-ADP concentration, and the average values were 59 ± 5 and 41 ± 5% for fast and slow phases, respectively.

| Parameter | Value | Signal |
|-----------|-------|--------|
| ATP binding | \( K_{i} \) (\( \mu \text{M}^{-1} \text{s}^{-1} \)) | 1.4 ± 0.1^a | Pyreacin |
| | \( K_{i} (\text{ms}) \) | 620 ± 40^a | Pyreacin |
| | \( 1/K_{i} (\text{ms}) \) | 450 ± 70^a | Pyreacin |
| ADP binding | \( k_{5 \text{ fast}} (s^{-1}) \) | 5.4 ± 0.4^a | mant-ADP |
| | \( k_{5 \text{ slow}} (s^{-1}) \) | 4.7 ± 0.3^a | Pyreacin |
| | \( k_{6 \text{ fast}} (\mu \text{M}^{-1} \text{s}^{-1}) \) | 0.35 ± 0.12^a | mant-ADP |
| | \( k_{6 \text{ slow}} (s^{-1}) \) | 0.46 ± 0.06^a | Pyreacin |
| | \( k_{5 \text{ overall}} (\mu \text{M}) \) | 3.0 ± 0.5^a | mant-ADP |
| | Calculated from \( k_{5 \text{ fast}} \) and \( k_{5 \text{ slow}} \) | 0.36 | mant-ADP |
| Actin binding | \( k_{6 \text{ actin}} (s^{-1}) \) | 2.4 ± 0.1^a | mant-ADP |
| | \( k_{6 \text{ slow}} \) | 0.16 ± 0.03^a | mant-ADP |
| | \( K_{i} (\text{mM}) \) | 2.3 ± 0.14^a | mant-ADP |
| | Calculated from \( k_{6 \text{ fast}} \) and \( K_{i} \) | 0.029 | mant-ADP |

The equilibrium dissociation constant for ADP binding to ATM1 of the slow phase (\( K_{s \text{ slow}} \)) is calculated as \( K_{s \text{ slow}} = k_{s \text{ fast}}^{2}/k_{s \text{ slow}} \) and is 0.029 (0.16/3.5 s^{-1}). The equilibrium dissociation constant for ADP binding to ATM1 of the fast phase (\( K_{f \text{ fast}} \)) is calculated as \( k_{s \text{ fast}}^{2}/k_{s \text{ slow}} \) and is 1.0 \( \mu \text{M} \) (2.4/3.5s^{-1}). The overall equilibrium dissociation constant for ADP binding to ATM1 (\( K_{\text{overall}} \)) is calculated as \( k_{s \text{ fast}}^{2}/(1 + K_{s \text{ slow}}) \) (35, 55) and is 0.029 \( \mu \text{M} \) (Table 4).

**Actin-ATM1 Interaction in the Presence of ATP**—To determine the affinity of ATM1 for actin in the presence of ATP, actin co-sedimentation assays (29) were performed under physiological ionic conditions. Fig. 7 shows the fraction of ATM1-MD bound to actin as a function of actin concentration. The data were fit to a hyperbola to determine the actin affinity in the presence of ATP. The equilibrium dissociation constant for actin binding to ATM1 in the presence of ATP (\( K_{a} \)) is 4.5 ± 0.7 \( \mu \text{M} \) (Table 4). This value is similar to that of animal myosin III (29), which is known to have high affinity for actin. The maximum percentage bound to actin was 90 ± 4.5%, showing that ATM1 is a high duty ratio (~90%) myosin.

**Actin-ATM1 Interaction in the Absence of ATP**—The affinity of ATM1 for actin in the absence of ATP (strong binding state) was measured using pyreacin (29, 75). The transients were fit to a single exponential function at each actin concentration (Fig. 8A, inset). The association rate constant linearly increased with actin concentration to yield the second-order rate con-
Enzymatic Properties of Class VIII Myosin

FIGURE 7. Affinity of ATM1 for actin in the presence of ATP. The affinity of ATM1-MD for actin filament was measured using the actin co-sedimentation assay. 1 μM ATM1-MD was equilibrated in the presence of various concentrations of actin, 2 mM ATP, and the ATP regeneration system, and the reaction mixture was subsequently centrifuged at 25 °C. The fraction of ATM1-MD bound to actin was quantified by performing densitometry on Coomassie-stained gels following SDS-PAGE. The plot of the fraction bound (closed circles) as a function of actin concentration was fit to a hyperbola to determine the affinity for actin (K_d of 4.5 ± 0.7 μM). The value after ± indicates S.D. of the regression curve.

FIGURE 8. Actin binding to and dissociation from ATM1 in the absence of ATP. A, observed rate constants of the actin binding to ATM1-MD (k_μM) were plotted against pyrene-actin concentrations. The concentration of ATM1-MD was kept at one-fifth of the pyrene-actin concentration. Linear fits to the data sets yielded second-order actin binding rate constants of 8.0 ± 0.6 μM⁻¹ s⁻¹ (k_μM). The value after ± indicates S.D. of the regression line. Inset, 0.4 μM ATM1-MD was mixed with 2 μM pyrene-actin (in final concentration). The transient is the average of eight separate recordings, and the red line is a single exponential fit, which yielded a rate a constants of 18.4 s⁻¹. B, rate of the dissociation of acto-ATM1. 0.2 μM pyrene-ATM1-MD was mixed with 5 μM unlabeled actin in the absence ATP (final concentration). The red line is a single exponential fit, which yielded a rate constant of 0.0048 s⁻¹ in the example shown. The averaged values from eight independent assays was 0.0057 ± 0.001 s⁻¹.

stant (k_d) of 8.0 ± 0.6 μM⁻¹ s⁻¹ (Fig. 8A and Table 4). The intercept of Fig. 8A yields the acto-ATM1-MD dissociation rate constants (k_d) but is subject to a large uncertainty when it is low (34, 35), so dissociation was measured directly by competition with unlabeled actin filaments (Fig. 8B). The observed rates were fit to a single exponential function, which yielded a rate constant of 0.0057 ± 0.001 s⁻¹ of k_d (Fig. 8B and Table 4). The equilibrium dissociation constant for actin binding to ATM1 in the absence of ATP (K_d) is calculated as k_d/k_μM and is 0.66 nM (0.0057 s⁻¹/8.0 μM⁻¹ s⁻¹, Table 4). This value is lower by a factor of 50 than that of animal skeletal myosin II (80) and by a factor of 10 than that of animal myosins III and VII that are known to have high affinity for actin (29, 35, 75, 81). This indicates that ATM1 binds actin with very high affinity in the strong binding state.

Expression Pattern of ATM1 in Arabidopsis—To determine the tissue-specific expression of ATM1, we created a transgenic Arabidopsis plant that expressed a GUS fusion protein under the transcriptional control of the ATM1 promoter. GUS staining indicated that ATM1 was abundantly expressed in seedlings, apices of shoots, shoots, and flowers (Fig. 9, A–D) but not in pollen (Fig. 9E).

Localization of ATM1 in Arabidopsis—In earlier studies using an antibody against a peptide corresponding to a tail domain of ATM1, it was suggested that ATM1 is localized to the plasmodesmata and newly formed cell walls in root cells of maize and Arabidopsis (22). The same antibody was used to show the localization of ATM1 on plastid (82). Moreover, when the GFP-fused tail domain of ATM1 was expressed in Arabidopsis, it was localized to the plasma membrane and plasmodesmata (23, 83). To obtain accurate information about the localization of ATM1, we used GFP-fused full-length ATM1 (GFP-ATM1) in this study because tail domain expression sometimes acts as a dominant negative, leading to artifactual localization (84). First, GFP-ATM1 was transiently expressed in protoplasts of cultured Arabidopsis cells and was observed using a high speed confocal laser scanning microscope (Fig. 10, A–C). Fluorescent dots were observed at the cell cortex (Fig. 10, A and B), similar to the results of previous studies using the GFP-fused tail domain of ATM1 (23). However, in contrast to other studies, punctate structures were present on filamentous structures such as actin bundles (Fig. 10B). To ascertain that this structure was formed by F-actin, Lifeact-TagRFP was expressed together with GFP-ATM1. ATM1 was co-localized with Lifeact-TagRFP at the cell cortex (Fig. 10C), indicating that ATM1 associated with F-actin at the cell cortex. Live imaging showed little movement of the dots on F-actin (supplemental Movie S1). This is consistent with the results of the in vitro actin-gliding assays, which showed that ATM1 is an extremely slow myosin (Fig. 3).

Next, GFP-ATM1 was expressed in Arabidopsis plants (Fig. 10, D–G). To avoid artifacts arising from differences in expression level and pattern, a construct expressing GFP-ATM1 under the control of the native ATM1 promoter was transformed into an Arabidopsis ATM1-knock-out mutant (atm1). Fig. 10D shows the expression of GFP-ATM1 in epidermal cells of root tip meristems. GFP-ATM1 was localized to newly formed cell plates, consistent with another study (22). In a growing elongating zone of epidermal cells, GFP-ATM1 was observed as fluorescent dots on filamentous structures (Fig. 10E) similar to protoplasts (Fig. 10B). To determine whether these filamentous structures were composed of F-actin, we treated cells with cytoskeletal inhibitors. The filamentous structures did not form in the presence of latrunculin B (Fig. 11B) but persisted when the cells were exposed to oryzalin (Fig. 11A). These data confirm that the filamentous structures consisted of F-actin. Live imaging showed little movement of the fluorescent ATM1 dots on F-actin (supplemental Movie S2), consistent with the results of the in vitro actin-gliding assays. The expression of GFP-ATM1 in epidermal cells of cotyledons
is shown in Fig. 10, F and G. Focusing on the center of the cell, ATM1 was localized to the punctate structures on plasma membranes that border neighboring cells (Fig. 10F, arrowheads), supporting an earlier observation that an anti-ATM1 antibody stains the plasmodesmata (85). ATM1 was also localized to structures presumed to be plastids (Fig. 10F, arrows), consistent with the results of a published study (82). Focusing on the subcortical region of the cell (Fig. 10G), ATM1 was observed at filamentous structures, most likely F-actin.

**DISCUSSION**

**ATM1 Exhibits Low Enzymatic Activity and High Affinity for Actin**—In this study, we reported the first analysis of the enzymatic properties of plant-specific class VIII myosin. We showed that actin-sliding velocity (Fig. 3) and actin-activated ATPase activity of *Arabidopsis* class VIII myosin, ATM1 (Fig. 2), were lower by a factor of 10–100 compared with those of class plant-specific XI myosins (15, 17, 18) and were similar to those of animal slow myosins (30, 51, 52). The affinity of ATM1 for actin was very high both in the presence (Fig. 7) and absence (Fig. 8) of ATP. However, it must be noted that the kinetics might be different due to phosphorylation or other post-translational modifications to the myosin.

**Slow ADP Dissociation Accounts for Low Velocity, Low Enzymatic Activity, and High Duty Ratio**—The dissociation rates of ADP from myosins are accelerated by actin binding, and the acceleration ($k_{+5}/k_{-5}$) differs between myosins. The value of the acceleration for fast class XI myosin is ~1000-fold (19) and that for slow animal myosins is only 1–10-fold (28, 33). The value for ATM1 was 2.3-fold (Table 4) showing that, similar to slow animal myosins, dissociation of ADP was not markedly influenced by actin binding.

**ATM1 Has the Kinetic Properties of a Tension Sensor**—We have shown in this study that ATM1 has two ADP states, open ADP pocket and closed ADP pocket. These two ADP states have been observed only in slow myosins but not fast myosins (29, 35, 54, 55, 79). Nyitrai and Geeves (86) suggested that the closed and open ADP states are in equilibrium in all myosins and that the equilibrium was dependent on the myosin types; almost all the ADP state are in the open state for fast myosins, whereas a considerable portion of ADP states are in the closed state for slow myosins. Thus two ADP states (the closed ADP pocket) can be observed only in slow myosins.

The swinging lever arm model proposes that the large free energy associated with P$_i$ release drives the power stroke of the cross-bridge (a swing of the converter and neck of the cross-bridge) to generate force. In some slow myosins, this swing is followed by a further swing of the converter and neck of the cross-bridge in the same direction as the power stroke when ADP is released (87–89). This additional swing concomitant with ADP release has been observed only in slow myosins but not fast myosins. Geeves *et al.* (26) suggested that this additional swing of the neck must be complete before the ADP binding pocket opens to allow ADP to escape. Such a mechanism provides a simple, elegant way of coupling ADP release to the load on the head because the load inhibits the swing and then ADP release. Thus, slow myosins in which a considerable por-
Enzymatic Properties of Class VIII Myosin

![FIGURE 10. Subcellular localization of GFP-ATM1 in protoplasts and Arabidopsis plants. A–C, GFP fused to the N terminus of full-length ATM1 was transiently expressed in protoplasts prepared from suspension cultures of Arabidopsis cells. A, focusing on the center of the cell. B, focusing on subcortical region of the cell. C, co-expression of GFP-ATM1 and Lifeact-TagRFP shows that ATM1 is co-localized with actin. D–G, GFP fused to the N terminus of full-length ATM1 was expressed under the control of the native promoter of ATM1 in Arabidopsis plants. D, epidermal cells of root apical meristems. ATM1 is localized at the filamentous structures. E, epidermal cells in a growing root. ATM1 is localized at the filamentous structures. F, epidermal cells of the cotyledon. Focusing on middle of the cells. G, epidermal cells of the cotyledon. Focusing on subcortical region of the cell. ATM1 is localized at the filamentous structures. A–G, scale bars, 10 μm.](image)

![FIGURE 11. Effects of cytoskeletal inhibitors on ATM1 localization in epidermal cells in growing roots. A, treatment with 10 μM oryzalin. B, 2 μM latrunculin. C, 0.1% DMSO (control). Scale bar, 0.1 mm. Filamentous localization of ATM1 disappeared when treated with the actin-depolymerizing agent, latrunculin B (B) but were present following treatment with Oryzalin, a microtubule depolymerizing agent (A).](image)

Expression of GUS under the control of the ATM1 promoter in Arabidopsis suggests that ATM1 is abundantly expressed in seedlings, apices of shoots, shoots, and flowers but not in pollen (Fig. 9). This result
suggests that ATM1 functions in all tissues during all stages of the plant’s life cycle.

Earlier studies using an antibody raised against a peptide corresponding to a tail domain of ATM1 suggested that ATM1 is localized to plasmodesmata, plasma membrane of newly formed cell walls, and plastids (22, 82). This was also supported by live cell imaging using the GFP-fused tail domain of ATM1 (23, 83). In this study, we further examined the localization of ATM1 in Arabidopsis using GFP-fused full-length ATM1 (GFP-ATM1). Furthermore, to avoid artifacts arising from differences of expression levels and pattern, GFP-ATM1 was expressed using the native promoter of Arabidopsis ATM1 in an ATM1-knock-out mutant (atm1). Consistent with the studies cited above, GFP-ATM1 was localized to the plasmodesmata (Fig. 10F), plasma membrane of newly formed cell walls (Fig. 10D), and plastids (Fig. 10F). However, in contrast to other studies, we observed that ATM1 was primarily localized to F-actin at the cell cortex in several tissues (Figs. 10, F and G, and 11). This was also confirmed by analysis of protoplasts prepared from suspension cultures of Arabidopsis cells (Fig. 10, A–C). ATM1 showed little movement on F-actin at the cell cortex (supplemental Movies S1 and S2), consistent with the results of the in vitro actin gliding assays.

Taken together, our enzymatic and localization studies suggest that in Arabidopsis class VIII myosin, ATM1 functions at various intracellular structures such as cell cortex, newly formed cell wall, plasmodesmata, and plastids as a tension sensor/generator.

Acknowledgments—We thank RIKEN Bio Resource Center (Tsukuba, Japan) for providing plasmids containing full-length cDNAs of Arabidopsis ATM1 (AT3G19960) and Arabidopsis CAM3 (AT3G58800), which were developed by the plant genome project of RIKEN Genomic Sciences Center (24, 25). We also thank T. Nakagawa (Center for Integrated Research in Science, Shimane University, Japan) for providing the binary vectors. We thank Dr. T. Ueda, Dr. T. Uemura, Dr. C. Saito, Dr. H. Abe, K. Shoda, E. Fursyama, K. Fukaya, R. Kish, and E. Matsumoto of the Nakano Laboratory for their assistance and suggestions.

REFERENCES
1. Odronitz, F., and Kollmar, M. (2007) Drawing the tree of eukaryotic life based on the analysis of 2,269 manually annotated myosins from 328 species. Genome Biol. 8, R196
2. Reddy, A. S., and Day, I. S. (2001) Analysis of the myosins encoded in the recently completed Arabidopsis thaliana genome sequence. Genome Biol. 2, RESEARCH0024
3. Tominaga, M., Kimura, A., Yokota, E., Haraguchi, T., Shimmen, T., Yamamoto, K., Nakano, A., and Ito, K. (2013) Cytoplasmic streaming velocity as a plant size determinant. Dev. Cell 27, 345–352
4. Avisar, D., Prokhnevsky, A. I., Makarova, K. S., Koonin, E. V., and Dolja, V. V. (2008) Myosin XI-K is required for rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in leaf cells of Nicotiana benthamiana. Plant Physiol. 146, 1098–1108
5. Hashimoto, K., Igarashi, H., Mano, S., Nishimura, M., Shimmen, T., and Yokota, E. (2005) Peroxosomal localization of a myosin XI isoform in Arabidopsis thaliana. Plant Cell Physiol. 46, 782–789
6. Li, J. F., and Nebenfuhr, A. (2007) Organelle targeting of myosin XI is mediated by two globular tail subdomains with separate cargo binding sites. J. Biol. Chem. 282, 20593–20602
7. Reisen, D., and Hanson, M. R. (2007) Association of six YFP-myosin XI-tail fusions with mobile plant cell organelles. BMC Plant Biol. 7, 6
8. Avisar, D., Abu-Abied, M., Belaussov, E., Sadot, E., Hawes, C., and Sparkes, I. A. (2009) A comparative study of the involvement of 17 Arabidopsis myosin family members on the motility of Golgi and other organelles. Plant Physiol. 150, 700–709
9. Peremyslov, V. V., Mockler, T. C., Filichkin, S. A., Fox, S. E., Jaiswal, P., Makarova, K. S., Koonin, E. V., and Dolja, V. V. (2011) Expression, splicing, and evolution of the myosin gene family in plants. Plant Physiol. 155, 1191–1204
10. Prokhnevsky, A. I., Peremyslov, V. V., and Dolja, V. V. (2008) Overlapping functions of the four class XI myosins in Arabidopsis growth, root hair elongation, and organelle motility. Proc. Natl. Acad. Sci. U.S.A. 105, 19744–19749
11. Sparkes, I. (2011) Recent advances in understanding plant myosin function: life in the fast lane. Mol. Plant 4, 805–812
12. Ueda, H., Yokota, E., Kutsuna, N., Shimada, T., Tamura, K., Shimmen, T., Hasezawa, S., Dolja, V. V., and Hara-Nishimura, I. (2010) Myosin-dependent endoplasmic reticulum motility and F-actin organization in plant cells. Proc. Natl. Acad. Sci. U.S.A. 107, 6894–6899
13. Ojanga, E. L., Jarve, K., Paves, H., and Truve, E. (2007) Arabidopsis thaliana myosin XIK is involved in root hair as well as trichome morphogenesis on stems and leaves. Protoplasma 230, 193–202.
14. Yokota, E., and Shimmen, T. (1994) Isolation and characterization of plant myosin from pollen tubes of lily. Protoplasma 177, 153–162
15. Tominaga, M., Kojima, H., Yokota, E., Orii, H., Nakamori, R., Katayama, E., Anson, M., Shimmen, T., and Oiwa, K. (2003) Higher plant myosin XI moves processively on actin with 35 nm steps at high velocity. EMBO J. 22, 1263–1272
16. Yamamoto, K., Kikuyama, M., Sutoh-Yamamoto, N., and Kamitsubo, E. (1994) Purification of actin based motor protein from Chara corallina. J. Biochem. 107, 175–180
17. Hachikubo, Y., Ito, K., Schiefelbein, J., Manstein, D. J., and Yamamoto, K. (2007) Enzymatic activity and motility of recombinant Arabidopsis myosin XI, MYA1. Plant Cell Physiol. 48, 886–891
18. Ito, K., Cashiyama, T., Shimada, K., Yamaguchi, A., Awata Jy, Hachikubo, Y., Manstein, D. J., and Yamamoto, K. (2003) Recombinant motor domain constructs of Chara corallina myosin display fast motility and high ATPase activity. Biochem. Biophys. Res. Commun. 312, 958–964
19. Ito, K., Ikebe, M., Kashiyama, T., Mogami, T., Kon, T., and Yamamoto, K. (2007) Kinetic mechanism of the fastest motor protein, Chara myosin. J. Biol. Chem. 282, 19534–19545
20. Ito, K., Yamaguchi, Y., Yanase, K., Ichikawa, Y., and Yamamoto, K. (2009) Unique charge distribution in surface loops confers high velocity on the fast motor protein Chara myosin. Proc. Natl. Acad. Sci. U.S.A. 106, 21585–21590
21. Knight, A. E., and Kendrick-Jones, J. (1993) A myosin-like protein from a higher plant. J. Mol. Biol. 231, 148–154
22. Reichelt, S., Knight, A. E., Hodge, T. P., Baluska, F., Samaj, J., Volkmann, D., and Kendrick-Jones, J. (1999) Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall. Plant J. 19, 555–567
23. Golomb, L., Abu-Abied, M., Belaussov, E., and Sadot, E. (2008) Different subcellular localizations and functions of Arabidopsis myosin VIII. BMC Plant Biol. 8, 3
24. Seki, M., Carninci, P., Nishiyama, Y., Hayashizaki, Y., and Shinozaki, K. (1998) High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated CAP trapper. Plant J. 15, 707–720
25. Seki, M., Narusaka, M., Kamiya, A., Ishida, I., Satou, M., Sakurai, T., Nakajima, M., Enju, A., Akiyama, K., Oono, Y., Muramatsu, M., Hayashizaki, Y., Kawai, I., Carninci, P., Itoh, M., Ishii, Y., Arakawa, T., Shibata, K., Shinagawa, A., and Shinozaki, K. (2002) Functional annotation of a full-length Arabidopsis cDNA collection. Science 296, 141–145
26. Geeves, M. A., Perreault-Micale, C., and Coluccio, L. M. (2000) Kinetic analyses of a truncated mammalian myosin I suggest a novel isomerization event preceding nucleotide binding. J. Biol. Chem. 275, 21624–21630
27. El Mezgueldi, M., Tang, N., Rosenfeld, S. S., and Ostop, E. M. (2002) The kinetic mechanism of Myo1e (human myosin-IC). J. Biol. Chem. 277, 21514–21521
28. De La Cruz, E. M., Wells, A. L., Rosenfeld, S. S., Ostop, E. M., and Sweeney, J.
Enzymatic Properties of Class VIII Myosin

H. L. (1999) The kinetic mechanism of myosin V. Proc. Natl. Acad. Sci. U.S.A. 96, 13726–13731.

29. Dózsa, A. C., Ananthanarayanan, S., Moore, J. E., Burnside, B., and Yengo, C. M. (2007) Kinetic mechanism of human myosin IIIA. J. Biol. Chem. 282, 216–231.

30. Homma, K., Saito, J., Ikebe, R., and Ikebe, M. (2000) Ca2+-dependent regulation of the motor activity of myosin V. J. Biol. Chem. 275, 34766–34771.

31. Wang, F., Chen, L., Arcucci, O., Harvey, E. V., Bowers, B., Xu, Y., Hammer, J. A., 3rd, and Sellers, J. R. (2000) Effect of ADP and ionic strength on the kinetic and motile properties of recombinant mouse myosin V. J. Biol. Chem. 275, 4329–4335.

32. Trybus, K. M., Krementsova, E., and Freyzon, Y. (1999) Kinetic characterization of a monomeric unconventional myosin V construct. J. Biol. Chem. 274, 27448–27456.

33. De La Cruz, E. M., Ostap, E. M., and Sweeney, H. L. (2001) Kinetic mechanism and regulation of myosin VI. J. Biol. Chem. 276, 32373–32381.

34. Yang, Y., Kovács, M., Xu, Q., Anderson, J. B., and Sellers, J. R. (2005) Myosin VIII from Drosophila is a high duty ratio motor. J. Biol. Chem. 280, 32061–32068.

35. Henn, A., and De La Cruz, E. M. (2005) Vertebrate myosin VIII is a high duty ratio motor adapted for generating and maintaining tension. J. Biol. Chem. 280, 39665–39676.

36. Homma, K., and Ikebe, M. (2005) Myosin x is a high duty ratio motor. J. Biol. Chem. 280, 29381–29391.

37. Kovács, M., Wang, F., and Sellers, J. R. (2005) Mechanism of action of myosin X, a membrane-associated molecular motor. J. Biol. Chem. 280, 15071–15083.

38. Inoue, A., Saito, J., Ikebe, R., and Ikebe, M. (2002) Myosin IXb is a single-headed minus-end-directed processive motor. Nat. Cell Biol. 4, 302–306.

39. Haithcock, J., Billington, N., Choi, K., Fordham, J., Sellers, J. R., Stafford, W. F., White, H., and Forgacs, E. (2011) The kinetic mechanism of mouse myosin VIIIA. J. Biol. Chem. 286, 8819–8828.

40. Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maek, K., Tabata, R., Kawai, T., Tanaka, K., Niwa, Y., Watanabe, A., Nakamura, K., Kimura, T., and Ishiguro, S. (2007) Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Biosci. Biotechnol. Biochem. 71, 2095–2100.

41. Tian, G. W., Mohanty, A., Chary, S. N., Li, S., Paap, B., Drakakaki, G., Kopec, C. D., Li, J., Ehrhardt, D., Jackson, D., Rhee, S. Y., Raikhel, N. V., and Citovsky, V. (2004) High-throughput fluorescent tagging of full-length Arabidopsis gene products in planta. Plant Physiol. 135, 25–38.

42. Kodama, T., Fukui, K., and Kometani, K. (1986) The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified Malachite Green method for determination of inorganic phosphate. J. Biochem. 99, 1465–1472.

43. Kuhlman, P. A., and Bagshaw, C. R. (1988) ATPase kinetics of the myosin ATPase of the brain and skeletal muscle by31P-MRS. Magnes. Res. 1, 302–306.

44. De La Cruz, E. M., Ostap, E. M., and Sweeney, H. L. (2005) Magnesium, ADP, and actin binding linkage of myosin V: evidence for multiple myosin V-ADP and actomyosin V-ADP states. Biochemistry 44, 8826–8840.

45. Heissler, S. M., and Manstein, D. J. (2012) Functional characterization of the human myosin-7a motor domain. Cell. Mol. Life Sci. 69, 299–311.

46. Günther, T. (2006) Concentration, compartmentation and metabolic function of intracellular free Mg2+. Magnes. Res. 19, 225–236.

47. Murphy, E., Freudenrich, C. C., Levy, L. A., London, R. E., and Lieberman, M. (1989) Monitoring cytosolic free magnesium in cultured chicken heart cells by use of the fluorescent indicator Furaptra. Proc. Natl. Acad. Sci. U.S.A. 86, 2981–2984.

48. Raju, B., Murphy, E., Levy, L. A., Hall, R. D., and London, R. E. (1989) A fluorescent indicator for measuring cytosolic free magnesium. Am. J. Physiol. 256, C540–C548.

49. Petersen, A., Kristensen, S. R., Jacobsen, J. P., and Herder, M. (1990)31P-NMR measurements of ATP, ADP, 2,3-diphosphoglycerate and Mg2+ in human erythrocytes. Biochim. Biophys. Acta 1035, 169–174.

50. Weghuber, J., Dieterich, F., Froschauer, E. M., Svidovà, S., and Schweyen, R. J. (2006) Mutational analysis of functional domains in Msr2p, the mitochondrial Mg2+ channel protein of Saccharomyces cerevisiae. FEBS J. 273, 1198–1209.

51. Westerblad, H., and Allen, D. G. (1992) Myoplasmic Mg2+ concentration in Xenopus muscle fibres at rest, during fatigue and during metabolic blockade. Exp. Physiol. 77, 733–740.

52. Okada, K., Ishikawa, S., and Saito, T. (1992) Cellular mechanisms of vasopressin and endothelin to mobilize [Mg2+] in vascular smooth muscle cells. Am. J. Physiol. 263, C873–C878.

53. Altura, B. M., Zhang, A., Cheng, T. P., and Altura, B. T. (1993) Cocaine induces rapid loss of intracellular free Mg2+ in cerebral vascular smooth muscle cells. Eur. J. Pharmacol. 246, 299–301.

54. Delva, P., Pastori, C., Degani, M., Montesi, G., and Lechi, A. (2004) Catecholamine-induced regulation in vitro and ex vivo of intramyocyte ionized magnesium. J. Membr. Biol. 199, 163–171.

55. Tashiro, M., and Konishi, M. (1997) Basal intracellular free Mg2+ concentration in smooth muscle cells of guinea pig tenia cecum: intracellular calibration of the fluorescent indicator furaptra. Biophys. J. 73, 3358–3370.

56. Dai, L. J., and Quamme, G. A. (1992) Cyclic nucleotides alter intracellular free Mg2+ in renal epithelial cells. Am. J. Physiol. 262, F1100–F1104.

57. Singh, J., and Wisdom, D. M. (1995) Second messenger role of magnesium in pancreatic acinar cells of the rat. Mol. Cell. Biochem. 149, 175–182.

58. Iotti, S., and Malucelli, E. (2008) In vivo assessment of Mg2+ in human brain and skeletal muscle by31P-MRS. Magnes. Res. 21, 157–162.

59. Rijkers, G. T., and Griffioen, A. W. (1993) Changes in free cytoplasmic magnesium following activation of human lymphocytes. Biochem. J. 299, 373–377.

60. Yazaki, Y., Asukagawa, N., Ishikawa, Y., Ohta, E., and Sakata, M. (1988) Estimation of cytoplasmic free Mg2+ levels and phosphorylation potentials in mung bean root tips by31P-NMR spectroscopy. Plant Cell Physiol. 29, 919–924.

61. Thaler, M. (1991) Lichtabhängige Anderungen cytoplasmatischer Ionenaaktivitäten bei Eremosphaera viridis. Untersuchungen mit ionenselekti-
Enzymatic Properties of Class VIII Myosin

MAY 2, 2014 • VOLUME 289 • NUMBER 18

JOURNAL OF BIOLOGICAL CHEMISTRY

73. De La Cruz, E. M., Sweeney, H. L., and Ostap, E. M. (2000) ADP inhibition of myosin V ATPase activity. *Biophys. J.* 79, 1524–1529

74. Yengo, C. M., and Sweeney, H. L. (2004) Functional role of loop 2 in myosin V. *Biochemistry* 43, 2605–2612

75. Kambara, T., Komaba, S., and Ikebe, M. (2006) Human myosin III is a motor having an extremely high affinity for actin. *J. Biol. Chem.* 281, 37291–37301

76. Millar, N. C., and Geeves, M. A. (1983) The limiting rate of the ATP-mediated dissociation of actin from rabbit skeletal muscle myosin subfragment 1. *FEBS Lett.* 160, 141–148

77. Siemankowski, R. F., Wiseman, M. O., and White, H. D. (1985) ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proc. Natl. Acad. Sci. U.S.A.* 82, 658–662

78. Geeves, M. A., and Jeffries, T. E. (1988) The effect of nucleotide upon a specific isomerization of actomyosin subfragment 1. *Biochem. J.* 256, 41–46

79. Yang, Y., Kovács, M., Sakamoto, T., Zhang, F., Kiehart, D. P., and Sellers, J. R. (2006) Dimerized drosophila myosin VIIA: a processive motor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5746–5751

80. Cremo, C. R., and Geeves, M. A. (1998) Interaction of actin and ADP with the head domain of smooth muscle myosin: implications for strain-dependent ADP release in smooth muscle. *Biochemistry* 37, 1969–1978

81. Watanebe, S., Ikebe, R., and Ikebe, M. (2006) Drosophila myosin VIIA is a high duty ratio motor with a unique kinetic mechanism. *J. Biol. Chem.* 281, 7151–7160

82. Wojtaszek, P., Anielska-Mazur, A., Gabrys, H., Baluska, F., and Volkmann, D. (2005) Recruitment of myosin VIII towards plastid surfaces is root-cap specific and provides the evidence for actomyosin involvement in root osmosensing. *Funct. Plant Biol.* 32, 721–736

83. Sattarzadeh, A., Franzen, R., and Schmelzer, E. (2008) The *Arabidopsis* class VIII myosin ATM2 is involved in endocytosis. *Cell Motil. Cytoskeleton* 65, 457–468

84. Lapierre, L. A., Kumar, R., Hales, C. M., Navarre, J., Bhartur, S. G., Burnette, J. O., Provance, D. W., Jr., Mercer, J. A., Bähler, M., and Goldenring, J. R. (2001) Myosin vb is associated with plasma membrane recycling systems. *Mol. Biol. Cell* 12, 1843–1857

85. Avisar, D., Prokhnovsky, A. I., and Dolja, V. V. (2008) Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. *J. Virol.* 82, 2836–2843

86. Nyitrai, M., and Geeves, M. A. (2004) Adenosine diphosphate and strain sensitivity in myosin motors. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 359, 1867–1877

87. Jontes, J. D., Wilson-Kubalek, E. M., and Milligan, R. A. (1995) A 32° tail swing in brush border myosin I on ADP release. *Nature* 378, 751–753

88. Whittaker, M., Wilson-Kubalek, E. M., Smith, J. E., Faust, L., Milligan, R. A., and Sweeney, H. L. (1995) A 35-Å movement of smooth muscle myosin on ADP release. *Nature* 378, 748–751

89. Veigel, C., Wang, F., Bartoo, M. L., Sellers, J. R., and Molloy, J. E. (2002) The gated gait of the processive molecular motor, myosin V. *Nat. Cell Biol.* 4, 59–65

90. Purcell, T. J., Morris, C., Spudich, J. A., and Sweeney, H. L. (2002) Role of the lever arm in the processive stepping of myosin V. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14159–14164

91. Sakamoto, T., Wang, F., Schmitz, S., Xu, Y., Xu, Q., Molloy, J. E., Veigel, C., and Sellers, J. R. (2003) Neck length and processivity of myosin V. *J. Biol. Chem.* 278, 29201–29207

92. Li, F. Y., Chaigne-Delalande, B., Kanellopoulou, C., Davis, J. C., Matthews, H. F., Douek, D. C., Cohen, J. I., Uzel, G., Su, H. C., and Lenardo, M. J. (2011) Second messenger role for Mg²⁺ revealed by human T-cell immunodeficiency. *Nature* 475, 471–476