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Plant myosins

Review article

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Dedicated to the memory of the late Professor Noburo Kamiya (1913–1999)

Summary. Plant myosins are motor proteins that bind to the external surfaces of organelles and interact with the cytoskeletal protein actin (as actin microfilaments), which organizes and directs intracellular movement. Recent progress in physiological, biochemical, immunological, and genetical studies of plant myosin has revealed considerable information about the structures and functions of these important molecules. This article briefly reviews the history of plant myosin research, summarizes recent progress, and highlights directions for future research.

Keywords: Actin filament; Ca^{2+}; Calmodulin; Cytoplasmic streaming; Myosin; Phosphorylation.

Abbreviation: AF actin filament.

Introduction

Myosins form a class of motor proteins that are ubiquitous in eucaryotic cells, where they function by sliding along actin filaments (AFs). Whereas actin is highly conserved and recognized with relative ease in plant cells by several methods, myosins are much more diverse with 13 or 14 classes currently recognized on the basis of the characteristics of their heavy chains (Cope et al. 1996, Mermall et al. 1998). They do however conform to a basic pattern of a head domain toward the N-terminal end that provides the motor function and a tail which varies widely between different myosins. The myosin first recognized (from skeletal muscle) is now termed myosin II. The next recognized type, formerly known as unconventional myosin, myosin I, is now joined by myosins of classes III to XIII. They are classified by their similarity in the head domain, but the differences in the head domains are usually supplemented by even more marked differences in their tails. The myosin II tail forms a coiled coil along its entire length (coiled coils are formed by in register alpha-helices that show a predictable pattern of hydrophilic and hydrophobic residues within a heptad repeat). The resultant heavy chain dimers assemble (with their associated light chains) into thick, bipolar filaments, and the heads protruding at each end move actin filaments (AFs) towards the center of the thick filament. Myosin I forms no coiled coils, whereas other classes, including classes VIII and XI found in plants, are predicted to form coiled coils along only parts of their tails and not to aggregate beyond a dimer. Other tail domains may form links transmitting forces to plasma membranes or organelle membranes, a trait of particular relevance in plant cell organelle movement.

Myosins proved more difficult to recognize in plants than did actin. Research began with studies of cytoplasmic streaming and, although streaming is readily observed in many plant species, most knowledge of its mechanism has come from studies of characean algae. Early studies of plant myosins were mainly indirect

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because myosins were difficult to identify by structural or biochemical methods. Nonetheless, important insights were obtained which in many cases have been borne out by later, more direct studies using structural, biochemical, immunological, and molecular methods.

The pioneering studies of Kamiya and Kuroda (1956) showed that the motive force for characean cytoplasmic streaming is generated by active sliding of the flowing endoplasm over the inner surface of a stationary cortical layer (sol–gel interface). This focused the attention of botanists on the structures and processes at the sol–gel interface in characean cells. By light microscopy (Kamitsubo 1966, 1972) and electron microscopy (Nagai and Rebhun 1966), bundles of microfilaments were discovered at the interface, closely associated with the envelopes of chloroplasts anchored to the gel layer. Decoration with proteolytic fragments of skeletal muscle myosin indicated that these were AFs (Palevitz et al. 1974, Williamson 1974, Palevitz and Hepler 1975, Kersey and Wessells 1976, Kersey et al. 1976). Since it was known that muscle contraction is generated by a sliding between AFs and myosin, the involvement of myosin in cytoplasmic streaming was inevitably suggested (Kamiya 1981, 1986). In this review we summarize the characteristics of plant myosins that have been revealed by physiological, biochemical, immunological, and genetical studies.

Localization of myosin

In skeletal muscle, myosin is sensitively inhibited by SH-reagents such as N-ethylmaleimide (NEM), but actin is rather insensitive (Sibata-Sekiya and Tonomura 1975, Tonomura and Yoshimura 1962, Yamaguchi et al. 1973). By centrifuging characean cells, NEM can be applied either to the flowing endoplasm accumulated at the centrifugal end of the cell or to the gel layer containing the AFs that remained in situ along the cell. Since cytoplasmic streaming was inhibited by the NEM treatment of the flowing endoplasm but not by the treatment of the stationary gel layer containing the AFs, it was concluded that the flowing endoplasm contained an NEM-sensitive component, presumably myosin (Chen and Kamiya 1975). Williamson (1975) suggested that myosin is associated with organelles of the flowing endoplasm on the basis of intracellular-perfusion experiments. Taking advantage of the large size of characean cells, the natural cell sap in the vacuole can be replaced by intracellular perfusion (Tazawa 1964). When the vacuole was perfused with a medium containing the Ca^{2+} chelator EGTA, the tonoplast disintegrates (tonoplast-free cell) (Williamson 1975, Tazawa et al. 1976), and the chemical environment of the cytoplasmic components supporting streaming can be artificially controlled by intracellular perfusion. Upon depletion of ATP, streaming organelles stopped and formed strong linkages with the actin bundles. Williamson (1975, 1976) therefore speculated that a myosin-like component associated with endoplasmic organelles forms a rigor complex with the actin bundles in the absence of ATP. He later reported filamentous structures that were associated with the endoplasmic reticulum and suggested that they might contain myosin (Williamson 1979). Nagai and Hayama (1979) also found possible myosin-containing structures linking organelles and actin bundles in tonoplast-free cells depleted of ATP.

Reconstitution experiments also suggested that myosin was associated with organelles. Shimmen and Tazawa (1982) isolated endoplasmic organelles from internodal cells of *Chara australis* and introduced them into internodal cells of *Nitella axilliformis* whose endoplasm had been completely removed by intracellular perfusion. Chara organelles actively moved along the Nitella actin bundles in an ATP-dependent manner. Since this motility was maintained after washing the organelles, it was suggested that myosin is bound to organelles. Kohno and Shimmen (1988a) extended the evidence to higher plants by showing that organelles from lily pollen tubes also moved along actin bundles when introduced into internodal cells of characean algae. Nothnagel and Webb (1982) and Yoneda and Nagai (1988) also suggested an association of myosin with organelles, on the basis of their analysis of hydrodynamic equations for streaming. Kachar and Reese (1988) reported movement of endoplasmic reticulum along actin bundles in isolated Chara cytoplasm. Association of endoplasmic reticulum with actin filaments has been reported in various plant cells (Forde and Steer 1976, Goosen-de Roo et al. 1983, Hensel 1985, Quader et al. 1987, Lichtscheidl et al. 1990, Boevink et al. 1998).

Physiological studies

Kersey et al. (1976) showed that heavy meromyosin from rabbit muscle combines with actin filaments to form arrowhead structures that point in the direction opposite to that of cytoplasmic streaming. Characean

myosin sliding on AFs from their pointed ends (minus) to their barbed ends (plus) could therefore support streaming in the observed direction. The organelles of the lily pollen tube also slide along characean actin bundles in the same direction (Kohno et al. 1990). The sliding of plant myosin toward the barbed end of an AF is consistent with that of muscle myosin (Sheetz and Spudich 1983). Demembranated characean cell models either without tonoplast (Williamson 1975, Tazawa et al. 1976) or with permeabilized plasma membrane (Shimmen and Tazawa 1983) have elucidated many characteristics of cytoplasmic streaming. Since cytoplasmic streaming is caused by sliding of myosin associated with organelles along AFs, the velocity of streaming directly reflects the sliding velocity between myosin and AFs. Shimmen (1978) used tonoplast-free cells to show that the concentration of ATP giving half maximum velocity of streaming was between 70 and 90 μM. ADP, orthophosphate, pyrophosphate (Shimmen 1988), and sulphate (Shimmen et al. 1990) inhibited streaming in competition with ATP. Thus demembranated cells make it possible to analyze some enzymatic characteristics of myosin–actin interactions without isolating proteins. Work using demembranated cells also contributed to elucidating the regulation of cytoplasmic streaming by Ca^{2+}, which will be discussed later.

Biochemical studies

Protein purification gives the most direct identification and characterization of plant myosins. Myosins have been isolated from *Nitella flexilis* (Kato and Tonomura 1977), *Egeria densa* (Ohsuka and Inoue 1979), tomato fruit (Vahey et al. 1982), *Heracleum sosnowskyi* petiole (Sokolov et al. 1986), and pea tendril (Ma and Yen 1989). These experiments have not always proved reproducible in different laboratories, indicating the difficulties of isolating myosin from plants. Two major problems have to be overcome to isolate plant myosins: successful measurement of a myosin-specific activity to provide an assay and protection of myosin from attack by vacuolar proteases and phenolics. Isolated myosin has several ATPase activities, such as Mg^{2+}-ATPase, K^{+}-EDTA-ATPase, Ca^{2+}-ATPase, and actin-activated ATPase, but other ATPases and active phosphatases that occur in crude extracts make it very difficult to selectively measure myosin ATPase. A putative mung bean myosin was identified and partially purified with antibodies against animal myosin, but the final material lacked ATPase activity (Lin et al. 1994). Such problems were overcome by the development of an in vitro motility assay, where fluorescently labeled AFs slide on a glass surface coated with myosin (Kron and Spudich 1986). This assay, using the most basic and specific property of myosin, can be performed in the presence of other ATPases. Kohno et al. (1991) showed that skeletal muscle AF slid on glass surfaces coated with a crude extract of lily pollen tubes, indicating that the extract contained functionally active myosin. This assay is now widely used to monitor myosin activity throughout the isolation procedure (Kohno et al. 1992, Yokota and Shimmen 1994, Yamamoto et al. 1994, Higashi-Fujime et al. 1995).

The problems of myosin degradation and inactivation stem from the large central vacuole that exists in fully grown plant cells. When plant cells are homogenized, their compartmentation breaks down and cytoplasmic proteins are attacked by proteases and phenolics originally confined to the vacuole. Using lily pollen tubes at an early stage of growth, Kohno et al. (1992) succeeded in partially isolating a myosin which translocated AFs in a motility assay. However, this approach was not always completely successful. It was later found that myosin suffered partial proteolysis, probably due to proteases present even in vacuoles of young cells. By adding casein to the crude extract, Yokota and Shimmen (1994) succeeded for the first time in isolating intact myosin with sliding activity from a plant. With giant cells, another method to avoid the vacuole problem is to remove vacuolar sap by intracellular perfusion before starting the isolation procedure. Shimmen and Tazawa (1982) perfused the vacuole of 30 internodal cells of *C. australis* with an EGTA-containing medium to remove vacuolar sap and then collected endoplasmic organelles that would slide along Nitella actin bundles. Yamamoto et al. (1994) scaled up to about 2000 cells and succeeded in isolating myosin from *Chara corallina*. Vacuoles can be removed from smaller plant cells by strongly centrifuging protoplasts in medium containing Percoll to prepare "miniprotoplasts" (Lorz et al. 1981). Miniprotoplasts have proved useful for isolating several cytoplasmic proteins (Sonobe 1996), although this method has not been applied to myosin isolation.

Another effective strategy to separate myosin from other soluble proteins and vacuolar contents is selective cosedimentation with AFs. Myosin in the crude extract binds to exogenously added AFs in the absence

of ATP. After ultracentrifugation, bound myosin is released from AFs by adding ATP (Yokota and Shimmen 1994). Thus, myosin can be separated from proteases and phenolics at an early stage in the isolation. By such methods, myosins maintaining sliding activity have been isolated from lily pollen tubes (Kohno et al. 1992, Yokota and Shimmen 1994), *C. corallina* (Yamamoto et al. 1994, Higashi-Fujime et al. 1995), and cultured tobacco cells (Yokota et al. 1999b) and concentrated from a crude extract of Vallisneria leaf cells (Takagi et al. 1995).

The velocity with which some isolated myosins translocate AFs in an in vitro motility assay broadly reflects the velocity of cytoplasmic streaming in the source tissue (7.7 μm/s for pollen tubes, Yokota and Shimmen 1994; 25 μm/s for *C. corallina*, Yamamoto et al. 1994). Since AFs of skeletal muscle were used for the motility assay in both experiments, it is suggested that the sliding velocity is determined by myosin species, not by AFs. Further support comes from the demonstration that a pollen tube myosin containing a 170 kDa heavy chain (170 kDa myosin) translocates plant and muscle AFs at the same velocity (Igarashi et al. 1999), a velocity close to that of cytoplasmic streaming in pollen tubes.

Information about the different properties of various plant myosins is beginning to appear. Pollen tube 170 kDa and Chara 230 kDa myosin show different sliding velocities with AFs; antibodies raised against their heavy chains did not cross-react with each other (Yamamoto et al. 1994, Yokota unpubl. results), and their mechanism of Ca^{2+} regulation is also different (discussed below). Two myosins have been isolated from cultured tobacco cells: the one with a 170 kDa heavy chain was recognized by an antibody against the 170 kDa heavy chain of pollen tube myosin, whereas the 175 kDa heavy chain was not, and vice versa (Yokota et al. 1999b). A 175 kDa myosin was not detected in pollen tubes of tobacco (Yokota et al. 1999b) or lily (Yokota unpubl.).

Yamamoto et al. (1995) showed that Chara myosin bound to phospholipid vesicles, suggesting the presence of a hydrophobic domain and the likely basis for the early observations suggesting that Chara myosin is organelle-associated. Electron microscopy showed that Chara myosin has two heads with size and shape similar to those of muscle myosin II. The tail, however, is shorter than that of muscle myosin II and forms a globular structure at the distal end. It has been argued that the ultrastructural morphology of Chara myosin is similar to that of members of the myosin V group (Cheney et al. 1993b), which are thought to be involved in organelle transport (Cheney et al. 1993a), and Chara myosin may bind to organelles through the globular tail.

Immunological studies

Myosins have also been detected with antibodies, usually antibodies raised against myosin II from mouse 3T3 cells, skeletal muscle, smooth muscle, or slime mold. In immunoblotting, the antibodies recognize polypeptides with molecular masses of 110, 158, 170, 175, and 200 kDa in higher plants (Parke et al. 1986, Lin et al. 1989, Tang et al. 1989, Radford and White 1998), 110 and 200 kDa in *C. corallina* (Grolig et al. 1988; Lin et al. 1989, 1994), and 220–230 kDa in *Ernodesmis verticillata* (La Claire 1991). When used to localize myosin by immunofluorescence, the antibodies label small organelles, endoplasmic reticulum, nucleus, and plasma membrane (Tischendorf et al. 1987, Grolig et al. 1988, Tang et al. 1989, Heslop-Harrison and Heslop-Harrison 1989, La Claire 1991, Liebe and Quader 1994, Terasaki and Niitsu 1994, Braun 1996, Radford and White 1998), findings consistent with the inferred organelle association and a role in cytoplasmic streaming. Miller et al. (1995) analyzed pollen tube myosins with antibodies against myosin IA, IB, II, and V and suggested three kinds of myosin to exist: myosin I, which translocates the generative cell and vegetative nucleus; myosin V, which translocates smaller organelles; myosin II, which (with myosin I) translocates larger organelles. Others, however, stained the generative cell and vegetative nucleus with antibodies against myosin II (Tang et al. 1989, Heslop-Harrison and Heslop-Harrison 1989). The molecular data we review below suggests caution is required in diagnosing myosin types with current antibodies.

Studies which use antibodies against purified plant myosins have fewer uncertainties. An antibody against animal myosin II recognized neither 170 kDa pollen tube myosin nor Chara myosin (Yokota and Shimmen 1994, Yamamoto et al. 1994), but the putative 170 kDa mung bean myosin was recognized by other antibodies to muscle myosin II, and antibodies to mung bean myosin recognized muscle myosin II (Lin et al. 1994). Antibodies against the 170 kDa myosin of pollen tubes bind to various organelles (Yokota et al. 1995a, b), consistent with this myosin's

proposed involvement in cytoplasmic streaming (Yokota and Shimmen 1994).

The question arises whether antibodies raised against myosin II are recognizing a plant myosin II. Myosins isolated from *N. flexilis* (Kato and Tonomura 1977), *Egeria densa* (Ohsuka and Inoue 1979), and *H. sosnowskyi* (Sokolov et al. 1986) apparently form bipolar filaments, suggesting that a myosin II may exist in plant cells. However, it is difficult to envisage how bipolar filaments of myosin II would translocate organelles along AFs, since myosin II is a motor protein that induces contraction by forming bipolar thick filaments rather than by association with membrane-bound organelles. Additional possibilities are the following.

Plant myosins II have a domain that binds to organelles.

Plant myosins II have functions other than organelle translocation. La Claire (1991) suggested the possibility that the 220–230 kDa polypeptide that is recognized in the green alga *E. verticillata* by an antibody against slime mold myosin II is involved in wound-induced cytoplasmic contractions. Myosin's involvement in plasmodesmatal function has also been suggested (Radford and White 1998).

Antibodies against myosins II from animals may recognize myosins of other types in plant cells. Myosins certainly share some amino acid sequences in the head region (Cheney et al. 1993a, Cope et al. 1996), and type-specific antibodies are only specific within the range of myosins against which they have been tested directly or their epitope (if it is known) identified from gene sequences. The complete genome sequence of *Arabidopsis thaliana* is likely to settle the range of myosin types occurring in dicotyledons but will leave unanswered many questions of function.

Molecular studies

Knight and Kendrick-Jones (1993) used the conserved amino acid sequence (GESGAGKT) from the phosphate-binding loop of the ATP-binding site to identify a cDNA from *A. thaliana* that encoded a polypeptide related to myosin heavy chains. The ATM1 protein had a predicted molecular mass of 131 kDa. Its motor domain is recognizably similar to other myosin motor domains and can be fitted to the proposed 3-dimensional structure for myosin heads (Cope et al. 1996). Analyses of the head suggested, however, that ATM1 was distinct from the seven categories of myosins then recognized (Cheney et al. 1993), leading Knight and Kendrick-Jones (1993) to make it the first member of class VIII. The C-terminal parts of ATM1 differed significantly from those of other myosins. Four IQ-motifs, that provide binding sites for calmodulin or related proteins (Rhoads and Friedberg 1997) were followed by a short region predicted to form a coiled coil and a unique C-terminal domain of about 150 residues.

Kinkema and Schiefelbein (1994) identified a cDNA encoding a larger putative myosin heavy chain (MYA1) from *A. thaliana*. Its predicted molecular mass was 173 kDa and it contained 6 to 7 imperfect IQ-motifs followed by a relatively short coiled-coil domain and an apparently unique tail domain. Consideration of its primary structure led Kinkema and Schiefelbein (1994) to assign it to class V, a group of myosins including some implicated in intracellular transport processes such as Golgi vesicle trafficking. Current analyses of head domains (Cope et al. 1996) now place these plant proteins in a separate group (group XI) in recognition of their divergence from the members of class V.

Many of the biochemical and immunological studies of plant myosins that we discussed earlier found that antibodies to myosins II from animal cells will react with many plant polypeptides ranging in size from less than 100 kDa to about 200 kDa or higher in some algae. Such findings raise the question whether those cross-reactions really indicate the range of plant myosin sizes and whether using antibodies to myosins I and II can indicate whether myosins I and II exist in plant cells. Molecular studies, and in particular the sequencing of the genome of *A. thaliana*, provide a very informative background to such discussions.

Regarding the number of myosins, Kinkema et al. (1994) provided evidence for six myosin-like genes in *A. thaliana*: one as a complete cDNA clone (MYA2), two as incomplete cDNAs (ATM2, MYA3), and three as polymerase chain reaction products (PCR 1, PCR 11, PCR 43). The PCR products potentially encode about 300–350 amino acids that appeared related to the MYA1 sequence (i.e., the type XI myosin). Combined with previous reports (ATM1, Knight and Kendrick-Jones 1993; MYA1, Kinkema and Schiefelfein 1994), this suggested at least eight myosin-like genes exist. Moeps et al. (1993) described another partial cDNA and Plazinski et al. (1997) sequenced even shorter PCR fragments that, since they did not match existing myosins, suggested further Arabidopsis

myosin genes exist. Sequencing of the complete genome of *A. thaliana* is well advanced, with potential proteins identified for about half of the genome. Ten more complete genes for myosin-related proteins have been found by a BLAST search using a consensus sequence for the myosin head (PR00193 at BLOCKS; http://blocks.fhcrc.org/blocks/) in addition to the four full-length genes previously identified (Knight and Kendrick-Jones 1993, Kinkema and Schiefelbein 1994, Kinkema et al. 1994) (Genbank pID numbers 3269298, 5734787, 4455334, 3142302, 4887746, 4512706, 3776579, 3063460, 2924770, 2494118). The much larger number of Arabidopsis proteins in Genbank annotated as myosin-like are probably not genuine myosins because they lack highly conserved sequences including GESGAGKT and the conserved head epitope identified by Plazinski et al. (1997). The sizes of the apparently authentic myosin heavy chains range from 126 kDa to 197 kDa. Presumably further genes remain to be found in the rest of the genome as it is sequenced and annotated in the near future. *Arabidopsis thaliana* may, therefore, have over 20 myosin genes, so that there is nothing intrinsically implausible about antibodies recognizing multiple polypeptides with widely differing sizes. All those antigenically related polypeptides need not necessarily be true myosins, however, just as many proteins in the database labelled as myosin-like on local sequence similarities do not prove to be authentic myosins on further analysis.

While we have not undertaken the analysis required to assign all the myosins to groups VIII or XI (Cope et al. 1996), alignment by BLAST searches distinguishes 2 as closely similar in sequence and size to ATM1 and ATM2 (myosins VIII), whereas the other 8 appear more similar to the myosins XI. Class VIII and XI myosins have also been reported in *Zea mays* by Liu and Pesacreta (Genbank pIDs 4960051, 4885026, 4733891), and a myosin XI in *Chlamydomonas reinhardtii* by Mages et al. (Genbank pID 3342148). None of the Arabidopsis gene products are predicted to show the very extensive regions of coiled coils (Lupas et al. 1991; program available at http://www.ch.embnet.org/software/COILS_form.html) that characterize myosins II and none completely lacks coiled coils. Myosins I are the most familiar class of myosin lacking coiled coils, but Menzel and Vugrek (Genbank pIDs 2731702 and 2051981) reported *Helianthus annus* and *Acetabularia cliftonii* myosins that are not predicted to contain coiled coils. Their predicted protein products have a molecular mass of only 101 and 97 kDa, respectively. Clearly, it will be interesting to see whether genes encoding such small myosins or myosins encoding type II myosins with extensive coiled coils emerge in the rest of the Arabidopsis genome.

The molecular data raises many issues, only a few of which can yet be settled. The 170 and 175 kDa plant myosins with demonstrated in vitro motility seem likely to be from the myosin XI class, judging by sizes predicted from the molecular data. The four type VIII myosins in *A. thaliana* are distinctly smaller (126 to 131 kDa) and have yet to be purified and shown to support motility in vitro. Genetic approaches, such as mutagenesis or antisense techniques, offer alternative routes to functional analysis but may face problems with the large number of genes in *A. thaliana* if this is associated with functional redundancy.

Regulation of myosin by Ca^{2+}

Inhibition of cytoplasmic streaming by Ca^{2+} seems to be a general phenomenon in plants (Shimmen 1992, Williamson 1993). This provides a clear contrast with muscle contraction, where sliding between myosin and AFs is activated by Ca^{2+}. Most physiological studies on the Ca^{2+} regulation of cytoplasmic streaming have used members of the family Characeae. Upon generation of an action potential at the plasma membrane, the cytoplasmic streaming quickly stops and recovers gradually (excitation–cessation coupling; Tazawa and Kishimoto 1968). Extracellular Ca^{2+} is required for excitation–cessation coupling (Barry 1968). The Ca^{2+} indicator protein aequorin shows that cytoplasmic free Ca^{2+} increases during an action potential. Ca^{2+} concentration increased to an estimated 7 μM in *C. corallina* and 42 μM in *Nitella* sp. (Williamson and Ashley 1982). Ca^{2+} microinjected into the cytoplasm reversibly inhibits cytoplasmic streaming (Hayama and Tazawa 1980, Kikuyama and Tazawa 1982). From these observations, it was hypothesized that the rising Ca^{2+} concentration in the cytoplasm stops cytoplasmic streaming. To establish the Ca^{2+}-hypothesis, it is required to show that an appropriate quantitative relationship exists between the Ca^{2+} concentration and the velocity of cytoplasmic streaming. Early demonstrations that Ca^{2+} inhibits streaming in tonoplast-free cells (Williamson 1975) required unphysiologically high Ca^{2+} levels to achieve severe but still incomplete inhibition of streaming. In plasma-membrane-permeabilized Nitella cells, however, cytoplasmic streaming

was inhibited by 1–10 μM Ca^{2+} (Tominaga et al. 1983), consistent with the measured rises in cytoplasmic free Ca^{2+} being sufficient to stop streaming. The lower sensitivity of tonoplast-free cells perhaps results from a depletion of signal transduction components.

Tominaga et al. (1987) proposed from inhibitor experiments that Ca^{2+}-induced cessation of cytoplasmic streaming involved the Ca^{2+}-dependent phosphorylation of myosin (termed indirect myosin-linked Ca^{2+} regulation to distinguish it from direct regulation by a Ca^{2+}-binding light chain). Further circumstantial evidence supports this model. Sliding of skeletal muscle myosin on characean actin bundles is insensitive to Ca^{2+} (Shimmen and Yano 1986), indicating that the Ca^{2+} sensitivity of cytoplasmic streaming is associated with myosin and not with actin. Phosphorylation of a putative myosin light chain by calcium-dependent protein kinases and localization of these kinases along actin bundles have been reported (McCurdy and Harmon 1992a, b). Myosin purified from *C. corallina* is insensitive to Ca^{2+} (Yamamoto et al. 1994), showing that any myosin-linked Ca^{2+} regulation must be indirect. The demonstration of Ca^{2+}-dependent phosphorylation and accompanying inhibition with isolated characean myosin would firmly establish the myosin phosphorylation hypothesis.

The reconstituted movement of pollen tube organelles on characean actin bundles is inhibited by micromolar Ca^{2+}, suggesting that Ca^{2+} regulates pollen tube cytoplasmic streaming through myosin (Kohno and Shimmen 1988a, b). Yokota et al. (1999a) have now directly demonstrated that micromolar levels of Ca^{2+} inhibit both the sliding and actin-activated ATPase of the 170 kDa myosin of pollen tubes. In addition, it has been found that calmodulin is a light chain of myosin so it is possible that pollen tube myosin activity is inhibited by binding of Ca^{2+} to calmodulin as a light chain (direct myosin-linked Ca^{2+} regulation) rather than indirectly by Ca^{2+}-dependent phosphorylation. The IQ-motifs, predicted to support both Ca^{2+}-dependent and Ca^{2+}-independent calmodulin binding (Rhoads and Friedberg 1997), reported for myosin genes in higher plants (Knight and Kendrick-Jones 1993, Kinkema and Schiefelbein 1994, Kinkema et al. 1994) suggest that Ca^{2+} regulation via calmodulin may be a common phenomenon. Ca^{2+} regulation of myosin seems to have in vivo relevance in pollen tubes. Active cytoplasmic streaming is not observed at their tips, where high, potentially inhibitory Ca^{2+} concentrations have been reported (Reiss and Nobling 1986; Rathore et al. 1991; Miller et al. 1992; Pierson et al. 1994, 1996). Treatment of pollen tubes to decrease the Ca^{2+} concentration at the tip region induced active cytoplasmic streaming at the tip region and inhibited tip growth (Miller et al. 1992; Pierson et al. 1994, 1996). To what extent Ca^{2+} regulates tube growth via its effects on myosins remains to be determined.

Conclusions

The presence of plant myosin began to be seriously considered when the involvement of AFs in cytoplasmic streaming was established (Kamiya 1981, 1986). Various approaches are revealing a variety of plant myosins, and the complete profile of myosins in *A. thaliana* will soon be available. In vitro studies of isolated myosins, identification and localization of myosins by antibodies, and studies of myosin genes have proceeded with little overlap. We need to integrate these approaches by, for example, testing predictions from gene sequences by using in vitro motility assays to characterize the gene products, using sequence data to raise antibodies with more defined specificity than possessed by antibodies raised to animal myosins, and using genetic manipulations to probe myosin's function in vivo and in vitro. Myosin's involvement in processes other than organelle movement may await discovery. The very high velocities of movement characean myosins support may shed light on many questions regarding the submolecular events in force generation and sliding (Uyeda 1996).

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