J Plant Res (2007) 120:31–43
DOI 10.1007/s10265-006-0061-0

CURRENT TOPICS IN PLANT RESEARCH

The sliding theory of cytoplasmic streaming: fifty years of progress

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Received: 18 August 2006 / Accepted: 22 November 2006 / Published online: 25 January 2007

Abstract Fifty years ago, an important paper appeared in *Botanical Magazine Tokyo*. Kamiya and Kuroda proposed a sliding theory for the mechanism of cytoplasmic streaming. This pioneering study laid the basis for elucidation of the molecular mechanism of cytoplasmic streaming—the motive force is generated by the sliding of myosin XI associated with organelles along actin filaments, using the hydrolysis energy of ATP. The role of the actin–myosin system in various plant cell functions is becoming evident. The present article reviews progress in studies on cytoplasmic streaming over the past 50 years.

Keywords Actin · *Chara* · Cytoplasmic streaming · Myosin · *Nitella*

Sliding theory

Since its discovery by Corti (1774), cytoplasmic streaming (protoplasmic streaming) had been recognized by many botanists. However, the mechanism of motive force generation remained unknown. In 1956, a pioneering paper opened the door to the elucidation of the molecular mechanism of cytoplasmic streaming (Kamiya and Kuroda 1956). The opening sentence of the paper was: ''One of the basic questions concerning the mechanism of protoplasmic streaming is whether the motive force of the flow is located within the streaming protoplasm itself or whether the driving energy is produced in the protoplasmic system not involved in the streaming so that the moving protoplasm is driven passively.''

In characean cells, the sol endoplasm rotates in the same direction with a constant velocity (rotational streaming). This stable streaming is suitable for quantitative analysis. Ewart (1903) published a figure showing the streaming velocity to be highest in the inner layers of the endoplasm and lowest at the interface between the endoplasm and the ectoplasm. The figure also showed streaming in the cell sap, with the highest velocity at a site close to the endoplasm. This figure was based on the idea that the motive force is generated in the flowing endoplasm. Kamiya and Kuroda (1956) used cinematographic analysis to obtain an exact figure for the velocity distribution in cells of *Nitella flexilis*. In analyses using rhizoid and leaflet cells, they did not find any appreciable velocity gradient at the boundary (Fig. 1a). They expressed the situation as follows: ''plasmasol layer (endoplasm) is actually not streaming, but just slipping as a whole on the inner surface of the cortical layer''. The vacuole showed the velocity distribution of a sigmoid pattern (Fig. 1a).

Kamiya and Kuroda centrifuged an internodal cell and collected the flowing endoplasm at the cell end. A cell fragment lacking a large central vacuole was prepared by ligation. The distribution of the streaming velocity in the cell fragment was sigmoid (Fig. 1b). They concluded that ''the interaction of organized gel surface and sol phase produces the shearing force which brings about the interfacial slippage'' (sliding at the sol–gel interface; sliding theory). Because the viscosity of the endoplasm is high, it moves as a mass in

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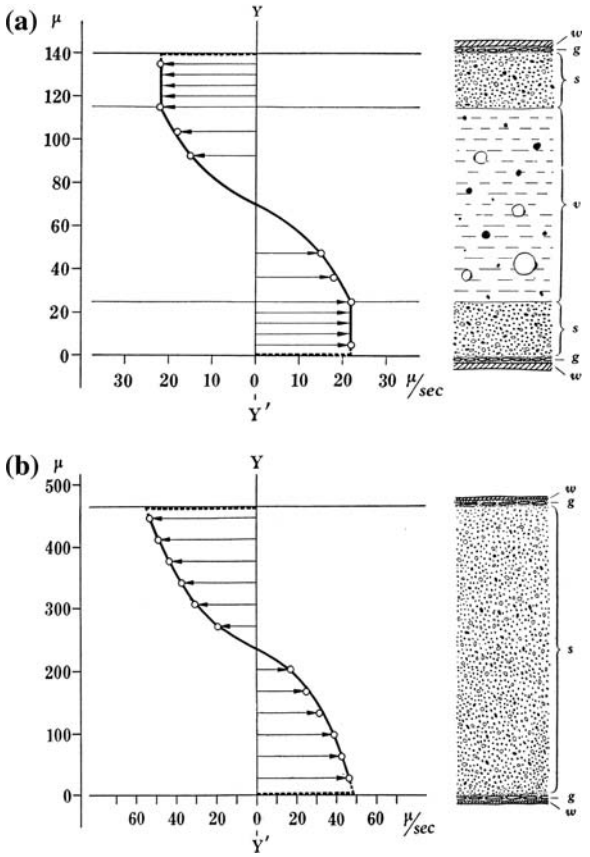

Fig. 1 Velocity distribution of cytoplasmic streaming in *N. flexilis* cells. **a** Cell containing a large central vacuole. **b** Cell lacking a large central vacuole. *w* cell wall, *g* gel ectoplasm, *s* sol endoplasm, *v* vacuole (from Kamiya and Kuroda 1956)

a cell having a large central vacuole, resulting in the absence of a velocity gradient in the endoplasm (Fig. 1a).

Bundle of microfilaments at the sol–gel interface

Kamiya and Kuroda (1956) suggested: ''What comes into focus concerning the rotational movement in *Nitella* is the mechanism causing the slippage between the sol–gel interface''. In leaflet and internodal cells of Characeae, chloroplasts are anchored to the gel ectoplasm (Fig. 2a). (In most plants, chloroplasts are mobile and move in response to light conditions). The sol–gel interface thus corresponds to the inner surface of the fixed chloroplasts.

It was then necessary to identify the structural entity at the gel surface responsible for motive force generation. Within a decade, two pupils of Noburô Kamiya found it. Kamitsubo (1966) discovered cables on the inner surface of chloroplast, by light microscopy. At the same time, Nagai and Rebhun (1966), using

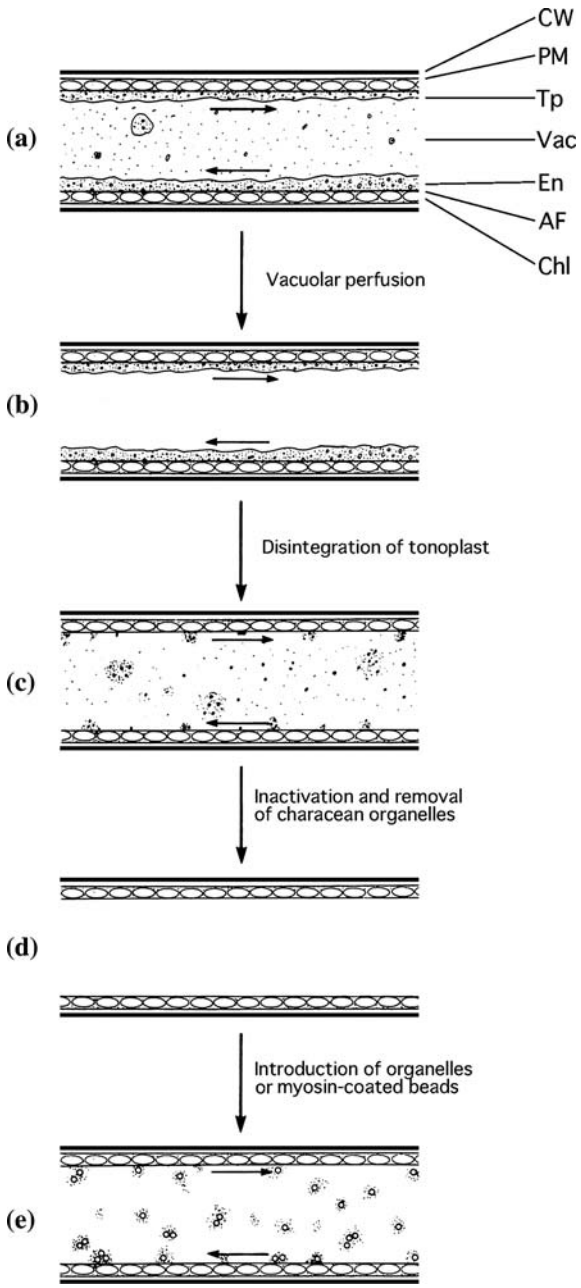

Fig. 2 Tonoplast-free cell of Characeae. **a** Intact cell. **b** Cell with artificial cell cap. **c** Tonoplast-free cell. **d** Cell lacking endogenous myosin. **e** Cell with exogenous organelles or myosin-coated beads. After both cell ends were amputated, the vacuole was perfused with an artificial medium (vacuolar perfusion). When the perfusion medium contained a Ca^{2+} chelator, the tonoplast disintegrated (**c** tonoplast-free cell). By the subsequent perfusion, the chemical composition in the cell is thoroughly controlled. Some of the endoplasmic organelles remained on the actin bundles and moved (not shown). Endogenous myosin is inactivated by N-ethylmaleimide (NEM) treatment and effuses out by strong perfusion (**d**). When organelles or myosin-coated beads are introduced by perfusion, the movement is reconstituted (**e**). *CW* cell wall, *PM* plasma membrane, *Tp* tonoplast, *Vac* vacuole, *En* endoplasm, *AF* actin filament, *Chl* chloroplast (modified from Shimmen 1988b)

electron microscopy, found these cables to be bundles of microfilaments.

Although the cables are located at the sol–gel interface, are they actually involved in generation of the motive force? When a small part of the gel layer was illuminated with strong light under a microscope, chloroplasts became detached from the gel layer and were swept away by the cytoplasmic streaming with the cables (Kamitsubo 1972; window technique). At the window, the cytoplasmic streaming was disturbed. When the cell was incubated for one to several days, the cables were regenerated, resulting in recovery of the streaming. This observation unequivocally showed the involvement of the cables in cytoplasmic streaming.

What are microfilaments?

Microfilaments were suggested to be actin filaments. At that time neither anti-actin antibody nor phalloidin was available. The only strategy available was demonstration of the arrowhead structure formed by application of fragments of skeletal muscle myosin, heavy meromyosin (HMM) or sub-fragment 1 (S1). Two groups, Williamson (1974) and Palevitz et al. (1974), showed the presence of microfilaments in the characean cells, which is decorated by HMM to form the arrowhead structure. Because the cells were completely disorganized, the intracellular localization of the actin filaments could not be shown. Palevitz and Hepler (1975) and Kersey and Wessells (1976) demonstrated the presence of actin cable at the sol–gel interface, the inner surface of chloroplasts. Kersey et al. (1976) further showed the polarity of the actin filaments in the cell. The orientation of the arrowheads indicated that cytoplasmic streaming occurs in the direction of the barbed end of the actin filaments. This direction was the same as that of myosin sliding along the actin filaments in skeletal muscle. Cytoplasmic streaming was stopped by treatment with cytochalasin B, which inhibits polymerization of actin filament (Williamson 1972; Bradley 1973), indicating involvement of the actin filaments in generation of the motive force.

Actin filaments are observed in a variety of plants (Staiger and Schliwa 1987), reflecting the ubiquitous occurrence of cytoplasmic streaming. In *Arabidopsis thaliana*, eight expressed functional actin genes are known. They are divided into two subclasses by their phylogeny and their expression pattern. *ACT2*, *ACT7*, and *ACT8* are strongly expressed in vegetative tissues, whereas *ACT1, ACT3, ACT4, ACT11*, and *ACT12* are preferentially expressed in reproductive tissues (Meagher et al. 1999).

Demembranated cell models

Demembranated cell models have extensively contributed to studies on the regulatory mechanism of cytoplasmic streaming. Two types of model have been developed.

Tonoplast-free cell

Tazawa (1964) developed a vacuole-perfusion method. After cutting both ends of an internodal cell of Characeae, the natural cell sap (vacuolar sap) is replaced with an artificial cell sap by intracellular perfusion (Fig. 2b). This makes it possible to control the chemical composition in the vacuole. The natural cell sap contains Ca^{2+} at mmol L^{-1} levels, which stabilizes the tonoplast. Williamson (1975) and Tazawa et al. (1976) added a Ca^{2+} chelator to the perfusion medium, leading to disintegration of the tonoplast (Fig. 2c). This disintegration causes disorganization of the cytoplasm. However, the actin bundles remain attached to the inner surface of the chloroplast layers, and organelles or their aggregates continue streaming. The intracellular chemical composition is thoroughly controlled by subsequent perfusion. Although most organelles effuse out, some remain on the actin bundles and move (not shown).

Permeabilized cell model

When the extracellular osmolarity is increased to a value higher than the intracellular osmolarity, plasmolysis is induced. If the plasmolyticum is suitable, protoplasts can survive. Shimmen and Tazawa (1983) induced plasmolysis in Characeae cells by using an ice-cooled medium containing a Ca^{2+} chelator. By plasmolysis under such severe conditions, the plasma membrane is irreversibly permeabilized but the tonoplast remains intact. Because the cytoplasm is sandwiched between the tonoplast and the perforated plasma membrane, it maintains its organization to some extent. In this model, the chemical composition of the cytoplasm is controlled by exchanging the extracellular medium.

ATP as an energy source

Although ATP had long been believed to be a direct energy source of cytoplasmic streaming, evidence for this was lacking until the development of demembranated cell models of Characeae. The ATP-dependent movement of organelles along actin bundles was directly demonstrated in tonoplast-free cells (Williamson

1975; Tazawa et al. 1976). Shimmen (1978) showed the hyperbolic relation between ATP concentration and the velocity. He also showed that ADP, orthophosphate, and pyrophosphate inhibited cytoplasmic streaming by competition with ATP, but AMP did not (Shimmen 1988a). Sulfate also inhibited cytoplasmic streaming by competition with ATP (Shimmen et al. 1990).

Contribution of characean actin bundles to studies on non-plant myosins

Characean actin bundles significantly contributed to studies on non-plant myosins. Kuroda and Kamiya (1975) prepared a cytoplasmic drop in which chloroplasts were rotating. Chloroplast rotation is thought to occur by the same mechanism as cytoplasmic streaming. After mechanical disruption of the surface membrane of the drop, chloroplast rotation was inhibited by treatment with *N*-ethylmaleimide (NEM). When HMM of the skeletal muscle was added, the chloroplasts resumed rotation. This was the first demonstration of the interaction between the characean actin bundles and the skeletal muscle myosin.

When exogenous organelles were introduced into the tonoplast-free cell, cytoplasmic streaming was reconstituted (Shimmen and Tazawa 1982). Thus, tonoplast-free cells can be a suitable material for reconstituting movement of foreign myosin. Another system was developed by Kuroda (1983), who cut open a *Chara australis* internodal cell parallel to its long axis. The cell wall was attached to the glass surface so that the inner surface of the chloroplasts was exposed. Using this system, Sheetz and Spudich (1983) succeeded in inducing movement of beads coated with HMM of skeletal muscle. Using tonoplast-free cells, Shimmen and Yano (1984) succeeded in inducing movement of beads coated with intact myosin of skeletal muscle.

The movement of beads coated with different myosin IIs was induced on characean actin bundles. Good correlation was observed between the actin-activated ATPase and the sliding velocity of different myosin IIs (summarized in Shimmen 1988b). Thus, the reconstituted movement was found to be a useful system for analyzing the sliding activity of myosins.

This reconstituted sliding provided an opportunity for quantitative analysis of Ca^{2+} regulation. In skeletal muscle, myosin sliding is induced by binding of Ca^{2+} to troponin integrated into the actin filaments (actin-linked Ca^{2+} regulation). The movement of beads coated with the skeletal muscle myosin on characean actin bundles was insensitive to Ca^{2+}. When the troponin–tropomyosin complex of the skeletal muscle was incorporated into the characean actin bundles, bead movement became Ca^{2+}-sensitive; movement occurred at high Ca^{2+} concentrations (Shimmen and Yano 1986). The actin-activated ATPase of scallop myosin is accelerated by binding of Ca^{2+} to myosin and that of *Physarum polycephalum* myosin is inhibited by binding of Ca^{2+} to myosin (direct myosin-linked Ca^{2+} regulation). These regulations were reconstructed on characean actin bundles (Vale et al. 1984; Kohama and Shimmen 1985). In smooth muscle, myosin is activated by phosphorylation via Ca^{2+}–calmodulin–light chain kinase (indirect myosin-linked Ca^{2+} regulation). Beads coated with the phosphorylated myosin moved on characean actin bundles, but those coated with the unphosphorylated form did not (Sellers et al. 1985).

From *Acanthamoeba castellanii*, Pollard and Korn (1973a, b) isolated an ATPase thought to be myosin. Although some biochemical characteristics were similar to those of muscle myosin, other characteristics were very different, e.g. the molecule was very small. The situation seemed intriguing, because only muscle-type myosins were known at that time. Later, Maruta and Korn (1977) isolated a muscle-type myosin from *A. castellanii*, and named the small myosin first isolated by Pollard and Korn (1973a) *Acanthoamoeba* myosin I and the other that they isolated *Acanthoamoeba* myosin II. Beads coated with myosin I moved along the characean actin bundles, showing this to be a real myosin (Albanesi et al. 1985). Organelles isolated from *A. castellanii* moved along the characean actin bundles, and this movement was inhibited by the antibody against myosin I but not by that against myosin II, suggesting organelle transport by myosin I (Adams et al. 1986). These findings led to the isolation of myosin I from a variety of cells.

Possible association of myosin with organelles

The involvement of actin filaments inevitably raised the possibility that myosin might be involved in generation of the motive force of cytoplasmic streaming. The presence of the putative myosin in the flowing endoplasm was suggested (Chen and Kamiya 1975, 1981; Kamitsubo 1981). The possible binding of putative myosin to organelles was first indicated by an experiment using tonoplast-free cells. Williamson (1975) found that organelles became strongly bound to actin bundles, when ATP was depleted by intracellular perfusion (inactive state). Upon addition of ATP, the organelles started their movement. This strong binding

of organelles to actin bundles in the absence of ATP (probably because of the action of an uncharacterized myosin-like component on the surface of organelles) reminded us of the so-called rigor cross-bridge of skeletal muscle myosin. Nagai and Hayama (1979) used electron microscopy to elucidate the morphological basis of the myosin-like component in tonoplast-free cells. They found that the endoplasmic organelles displayed a linkage with regularly arranged electron-dense materials. However, the identity of the linkage remained a question. Kachar and Reese (1988) found that a membrane structure thought to be the endoplasmic reticulum was moving on the actin bundles.

Shimmen and Tazawa (1982) functionally showed the binding of the myosin-like component with the organelles by an experiment in which cytoplasmic streaming was reconstituted in characean cells. After the natural cell sap of internodal cells of *Chara australis* had been removed by intracellular perfusion, the cytoplasm was isolated by squeezing the cell. In plant biochemistry, cell sap sometimes has detrimental effects because of low pH, high Ca^{2+} content, the presence of protease, or other factors. The cell sap was therefore removed beforehand. From the cytoplasm suspension, chloroplasts, nuclei, and the soluble components were removed by centrifugation. The organelle suspension thus prepared was introduced into a tonoplast-free cell of *Nitella axilliformis* by intracellular perfusion. *Chara* organelles actively moved along the *Nitella* actin bundles, indicating association of ''myosin'' with the organelles. Kohno and Shimmen (1988a) succeeded in moving organelles of the lily pollen tubes on the characean actin bundles, again supporting the association of ''myosin'' with the organelles of pollen tubes.

Purification of myosin

Although many botanists or biochemists have reported isolation of myosins from plant materials, all the work could not be reproduced. The most difficult aspect was measurement of myosin-specific activity in crude extracts of plant cells. Development of an in vitro motility assay was awaited in which fluorescent actin filaments could move on a glass surface coated with myosin. Yanagida et al. (1984) succeeded in observing single actin filaments labeled with fluorescent phalloidin. Kron and Spucich (1986) were able to induce sliding of a fluorescent actin filament on a glass surface coated with skeletal muscle myosin II. This ATP-dependent movement of the actin filaments on a myosin-coated glass surface is a specific activity of myosin.

Kohno et al. (1991) succeeded in inducing movement of muscle actin filaments on a glass surface coated with a crude extract of lily pollen tubes; this led to the isolation of plant myosins (Kohno et al 1992). The molecular weight of the myosin heavy chain was 120 kDa. However, the velocity of the actin filaments on the myosin-coated surface was much lower than that of the cytoplasmic streaming in the pollen tubes. This was later found to be a fragment of myosin formed during isolation by the action of proteases. Yokota and Shimmen (1994) succeeded in purifying intact myosin from lily pollen tubes by improving the purification procedure. One of the most important factors was addition of casein at the beginning of cell homogenization. Commercial inhibitors of protease could not prevent attack of myosin by proteases. Addition of casein enabled purification of the intact myosin molecule, whereas bovine serum albumin was ineffective. The molecular weight of the heavy chain was 170 kDa. The velocity in the in vitro motility assay was almost the same as that of cytoplasmic streaming in the pollen tubes, suggesting that this myosin is an entity involved in generation of the motive force of cytoplasmic streaming. The association of this myosin with organelles (Yokota et al. 1995a, b) also supports this possibility. When latex beads coated with the pollen-tube myosin were introduced into the tonoplast-free cells of *Nitellopsis obtuse*, ''streaming'' of the beads was induced along the actin cables (Yokota and Shimmen, unpublished). From cultured tobacco BY-2 cells, Yokota et al. (1999b) identified two myosins, 170 kDa myosin and 175 kDa myosin.

Yamamoto et al. (1994) succeeded in isolating myosin from *Chara corallina*, by using an in vitro motility assay. First, they removed the natural cell sap by intracellular perfusion (Shimmen and Tazawa 1982). When squeezing out the cell contents care was taken to prevent possible contamination with microorganisms attached to the cell wall. The molecular weight of the heavy chain of *Chara* myosin was 230 kDa.

Characterization of myosin

Gene analysis showed that both higher plant myosin (Tominaga et al. 2003) and *Chara* myosin (Morimatsu et al. 2000; Kashiyama et al. 2000) are types of myosin XI, the genes of which had been reported for *A. thaliana* (Reichelt and Kendrick-Jones 2000; Sellers 2000). The heavy chain of myosin XI is composed of a head domain, IQ motifs, α-helical coiled-coil domains, and a DIL domain (Fig. 3). The head domain converts the chemical energy of ATP to a mechanical force which

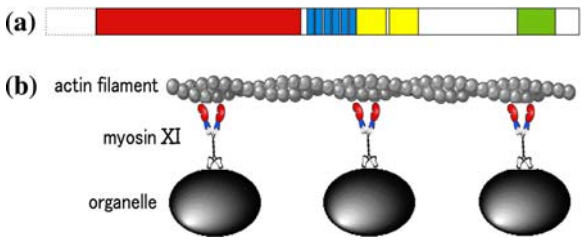

Fig. 3 Structure and function of higher plant myosin XI. *Red* head domain, *blue* neck region comprising six IQ motifs, *yellow* α-helical coiled-coil domain to form dimer, *green* DIL domain (modified from Shimmen and Yokota (2004)

causes sliding along the actin filaments. The presence of the α-helical coiled-coil domain suggests dimerization of heavy chains. Electron microscopy showed that myosin XI has two heads and a tail (Yamamoto et al. 1995; Tominaga et al. 2003). It is expected that the tail region of myosin XI is responsible for binding to organelles.

By single-molecule analysis, the higher plant myosin XI was shown to have high processivity (long distance sliding along an actin filament without detachment). This seems suitable for the function of myosin carrying a cargo along an actin rail. On the other hand, the movement of *Chara* myosin XI is non-processive (Awata et al. 2003; Kimura et al. 2003). Therefore, larger numbers of myosin molecules are needed to processively carry a cargo along an actin filament.

The velocity of cytoplasmic streaming varies among cell species; the fastest streaming, up to 100 μm s^{-1}, is observed in characean cells. Biophysicists are interested in the high speed of *Chara* myosin, anticipating that study of it may enable elucidation of the mechanism of actin–myosin sliding. The Yamamoto group is analyzing recombinant *Chara* myosin to elucidate the high-velocity molecular mechanism (Kashiyama et al. 2001; Ito et al. 2003; Seki et al. 2004).

To evaluate the involvement of actin filaments in physiological phenomena in plant cells, the effects of cytochalasin, latrunculin, bistheonellide, and phalloidin have been examined. Few strategies are available for myosin. Chen and Kamiya (1975) used NEM as an inhibitor of the putative myosin contained in the flowing endoplasm. Seki et al. (2003) showed that, in vitro, *Chara* myosin XI was far less susceptible to NEM than skeletal muscle myosin. It must be noted that NEM is a common SH-blocking reagent developed for protein chemistry and is not a specific myosin inhibitor.

When a target phenomenon of the plant cell was found to be inhibited by 2,3-butanedione monoxime (BDM), involvement of myosin was suspected. Tominaga et al. (2000) showed that BDM inhibits the activity of higher plant myosin XI in vitro. Inhibition of *Chara* myosin XI by BDM was also shown (Funaki et al. 2004). BDM has an inhibitory effect at a high concentration even in vitro. Because the concentration must inevitably be high in in vivo experiments, side effects are sometimes observed in organizations of actin filaments and microtubules (Tominaga et al. 2000). In addition to myosin XI, plant cells are equipped with myosin VIII (Sellers 2000). The effect of BDM on myosin VIII is not known, because this myosin has not yet been isolated.

An inhibitor developed for myosin II, ±blebbistatin (Straight et al 2003), did not inhibit cytoplasmic streaming in lily pollen tubes or tobacco BY-2 cells (Yokota, unpublished).

Association of myosin XI with organelles

The association of ''myosin'' with the organelles of plant cells has been repeatedly reported, on the basis of results from immunocytochemical analyses in which an antibody against myosin II from animals has usually been used. However, this antibody against myosin II does not recognize the myosin XI of higher plants and vice versa (Yokota and Shimmen 1994). The antibody against the *Chara* myosin XI does not recognize the myosin XI of higher plants (Yamamoto et al. 1994). Thus, the specificity of antibodies of myosins seems very high. One extreme example is that antibodies against two myosin XIs in tobacco cells (170 kDa myosin XI and 175 kDa myosin XI) do not cross-react with each other (Yokota et al. 1999b). Association of the myosin XI with organelles was demonstrated using the proper antibodies in higher plants (Yokota et al. 1995a, b) and in *Chara* (Morimatsu et al. 2000; Yamamoto et al. 2006). However, the organelles themselves have not been identified.

Hashimoto et al. (2005) prepared an antibody against the C-terminal region of MYA2, one of the myosin XIs of *A. thaliana*. The antibody recognized a polypeptide of 170 kDa, which was expected from the amino acid sequence deduced from the gene. The polypeptide interacted with actin filaments in an ATP-dependent manner, which is one of the functions of myosin. Immunolocalization revealed that MYA2 was co-localized with peroxisomes; it had been suggested that movement of these was acto-myosin-dependent (Jedd and Chua 2002). It was therefore suggested that MYA2 was a motor protein responsible for transport of peroxisome. The MYA2 gene was destroyed by inserting T-DNA into the intron. In the mutant, the distribution of peroxisome was the same as in the wild strain. Other

isoforms of myosin XI may be also involved in peroxisome transport, as discussed by Hashimoto et al. (2005). Both the growth and fertility of plants did not change with the mutation. Holweg and Nick (2004) had reported a loss-of-function mutant of MYA2 caused by T-DNA insertion. In this mutant, growth, fertility, and auxin transport were severely inhibited. This result was inconsistent with that of Hashimoto et al. (2005). However, the possibility was suggested of this mutant containing an additional deletion yet unidentified (Holweg, personal communication).

In *Closterium peracerosum-strigosum-littorale* complex, one of the myosin XIs is more highly expressed during sexual reproduction and this expression is cell-cycle dependent (Hamada et al. 2006). It seems that myosin XI is involved in organelle transport specific to the growth stages of the cells.

In yeast, myosin V is involved in the transport of organelles (Kacher et al. 2002; Fehrenbacher et al. 2003). Because the purpose of this article is to review organelle transport in plants, myosin V will not be discussed here.

Inhibition of cytoplasmic streaming by Ca^{2+}

When characean cells were stimulated using electric current or by mechanical shock, an action potential was found to be generated because of activation of Ca^{2+} and Cl^- channels (c.f. Shimmen et al. 1994; Tazawa and Shimmen 2001; Shimmen 1996). Upon generation of an action potential, cytoplasmic streaming stops transiently (Hill 1941; Sibaoka and Oda 1956; Kishimoto and Akabori 1959). The necessity of extracellular Ca^{2+} to stop the cytoplasmic streaming was first shown by Barry (1968). Cytoplasmic streaming (Kikuyama and Tazawa 1982) and the rotation of chloroplasts in the cytoplasmic drop (Hayama and Tazawa 1980) are reversibly inhibited by Ca^{2+} injected iontophoretically. The quantitative relationship between Ca^{2+} concentration and the velocity of cytoplasmic streaming was analyzed by using the permeabilized cell model of *N. axilliformis* (Tominaga et al. 1983). Addition of 1 μmol L^{-1} Ca^{2+} completely but reversibly inhibited cytoplasmic streaming. The influx of Ca^{2+} across the plasma membrane significantly increases upon generation of action potentials (Hayama et al. 1979). Williamson and Ashley (1982) found that free Ca^{2+} in the cytoplasm increased to 6.7 μmol L^{-1} in *C. corallina* and to 43 μmol L^{-1} in *Nitella* sp. upon generation of an action potential.

Tazawa and Kishimoto (1968) showed that cytoplasmic streaming stops at the peak of the action potential. Kikuyama et al. (1993) found that the cytoplasmic Ca^{2+} concentration began to increase at a very early stage of the action potential and that the streaming stopped after the Ca^{2+} increase. It was concluded that the cytoplasmic streaming is inhibited by Ca^{2+} upon generation of an action potential.

A brackish water charophyte, *Lamprothamnium succinctum*, regulates intracellular osmolality in response to changing extracellular osmolality (turgor regulation). When cells are subjected to hypotonic treatment, membrane depolarization and a decrease in membrane resistance are induced (Okazaki et al. 1984). At the same time, the rate of the cytoplasmic streaming falls to almost zero (Okazaki and Tazawa 1986). The hypotonic treatment increases the free Ca^{2+} in the cytoplasm, reflecting the Ca^{2+} sensitivity of cytoplasmic streaming (Okazaki et al. 1987).

Phosphorylation hypothesis of Characeae

Chara myosin XI has six IQ motifs (Kashiyama et al. 2000; Morimatsu et al. 2000), suggesting calmodulin to be a light chain. Awata et al. (2001) reported that calmodulin was present in the quickly-prepared myosin XI fraction. However, association of calmodulin with *Chara* myosin XI is yet to be demonstrated. The actin-activated ATPase of the *Chara* myosin XI was insensitive to Ca^{2+} (Yamamoto et al. 1994). Pharmacological study using the demembranated cell model suggested that Ca^{2+}-dependent protein phosphorylation is responsible for stoppage of the cytolasmic streaming (Tominaga et al. 1987). The motility of the myosin observed in the in vitro motility assay was inhibited by treatment with okadaic acid (inhibitor of protein phosphatase) or protein kinase C, but activated by treatment with staurosporine (inhibitor of protein kinase) (Morimatsu et al. 2002). This suggested that phosphorylation of myosin is responsible for Ca^{2+}-induced stoppage of the cytoplasmic streaming.

Ca^{2+} sensitivity is maintained in permeabilized models where the cytoplasm maintains its integrity (Tominaga et al. 1983). However, it is severely damaged in tonoplast-free cells in which the integrity of the cytoplasm has been injured (Hayama et al. 1979; Tominaga and Tazawa 1981). In the tonoplast-free cells, however, the Ca^{2+}-sensitivity is maintained, if the integrity of the cytoplasm is maintained (Tominaga et al. 1987). McCurdy and Harmon (1992a) reported that Ca^{2+}-dependent protein kinase is associated with the actin bundles and the organelles. They also reported that Ca^{2+}-dependent protein kinase phosphorylated a polypeptide recognized by an antibody against the light chain of gizzard myosin II (McCurdy and Harmon 1992b). However, it is unclear whether the

polypeptide is really a light chain of the *Chara* myosin XI. Thus, the mechanism of regulation of cytoplasmic streaming by Ca^{2+}-dependent phosphorylation in Characeae remains to be elucidated.

Ca^{2+}regulation in higher plants

Inhibition of the cytoplasmic streaming by Ca^{2+} has been also reported in higher plants: pollen tubes (Herth 1978; Kohno and Shimmen 1988b), stamen hair cells of *Tradescantia* sp. (Doree and Picard 1980), trichome cells of *Lycopersicon esculentum* (Wood et al. 1984), and mesophyll cells of *Vallisneria gigantea* (Takagi and Nagai 1986; Hayashi et al. 2003, 2006).

The reconstituted movement of the organelles of pollen tubes along the actin bundles of Characeae is inhibited by Ca^{2+} (Kohno et al. 1988a). Because the actin bundles of Characeae lack Ca^{2+}-sensitivity (Shimmen and Yano 1986), myosin associated with the pollen tube organelles was thought to be sensitive to Ca^{2+}. When the intracellular Ca^{2+} concentration of the pollen tubes was increased by using an ionophore, cytoplasmic streaming was inhibited. Increasing the Ca^{2+} concentration resulted in fragmentation of the actin filaments. Thus cytoplasmic streaming in the pollen tube may be inhibited by Ca^{2+} by both inhibition of myosin activity and fragmentation of the actin filament. Yokota et al. (2005) recently showed that higher plant villin fragments actin filaments in a Ca^{2+}-dependent manner.

A biochemical study has shown that the sliding activity of pollen tube myosin XI is inhibited by Ca^{2+}. It has also been shown that the light chain of myosin XI is calmodulin (Yokota et al. 1999a). This inhibition was also found for two myosin XIs of tobacco (Yokota et al. 1999b). Thus, the activity of higher plant myosin XI is inhibited by direct binding of Ca^{2+}.

Microtubules and cytoplasmic streaming

The motive force of cytoplasmic streaming is generated by the actin-myosin system in most plants. However, involvement of microtubules has been also reported in some algae. Kuroda and Manabe (1983) found that cytoplasmic streaming of *Caulerpa parvifolia* was inhibited by colchicine and *erythro*-9-[3-(2-hydroxynonyl)]adenine, an inhibitor of dynein. However, it was not inhibited by cytochalasin B, suggesting involvement of the microtubule-dynein system. The involvement of microtubules in the cytoplasmic streaming has been suggested in *Bryopsis plumosa* (Mizukami and Wada 1983; Menzel and Elsner-Menzel 1989) and *Dichotomosiphon tuberosus* (Maekawa et al. 1986).

Romagnoli et al. (2003) found that organelles isolated from the pollen tubes moved along microtubules in an ATP-dependent manner. They isolated a kinesin-related motor protein. Thus, the actin-myosin and microtubule-kinesin systems are both involved in organelle transport in the pollen tubes. The velocity of the kinesin-like motor protein is approximately 300 nm s^{-1}, whereas the velocity of myosin XI is more than ten times higher. Romagnoli et al. (2003) suggested that the microtubule system is involved in the short-range transport of organelles.

The cytoplasmic streaming of *Acetabularia mediterranea* is completely inhibited by cytochalasin (Koop and Kiermayer 1980). Koop and Kiermyer (1980) found that inhibitors of microtubules also inhibited movement of some organelles, but not others, in *A. mediterranea*. Thus, microtubules seem to be involved in the actin-based streaming. In root hair cells of *Hydrocharis dubia*, cytoplasmic streaming is inhibited by cytochalasin but not by propyzamide, indicating that the motive force is generated by the actin-myosin system (Tominaga et al.1997). Disruption of the microtubules inhibited recovery of actin organization and cytoplasmic streaming after inhibition with cytochalasin, suggesting the interaction of microtubules and actin filaments. Morphological study showed co-localization of these cytoskeletons (Tominaga et al. 1997).

When microtubules are destroyed, cytoplasmic streaming becomes sensitive to cytochalasin in *C. corallina* (Colling et al. 1996). Most microtubules are located just inside the plasma membrane (cortical microtubules), whereas the actin bundles responsible for the cytoplasmic streaming are located on the inner surface of chloroplasts (subcortical actin bundles). Thus, both cytoskeletons are spatially separated. Very few microtubules are colocalized with the sub-cortical actin bundles. Colling et al. (1996) suggested that tubulin or microtubule-associated proteins released by microtubule depolymerization interact with the actin bundles, making them sensitive to the cytochalasin treatment.

Photodinesis

In most plant cells, cytoplasmic streaming is always observed (primary streaming), whereas it is induced by a stimulus, for example light and chemicals, in *Vallisneria*, *Egeria*, and *Elodea* (secondary streaming). Induction of cytoplasmic streaming by light is called photodinesis.

The mechanism of photodinesis in *V. gigantea* mesophyll cells has been extensively studied by Takagi and Nagai. When cells were illuminated with red light, cytoplasmic streaming was induced; it was inhibited by far-red light. These results suggested involvement of phytochrome (Takagi and Nagai 1985). On the other hand, Seitz (1967) reported that blue light induced photodinesis in the epidermal cells of *Vallisneria spiralis*. The reason for this discrepancy remains unexplained.

When a Ca^{2+} chelator was added to the external medium, cytoplasmic streaming was induced even in the dark (Takagi and Nagai 1983). In the presence of a Ca^{2+} ionophore, cytoplasmic streaming was controlled by the extracellular Ca^{2+}: cytoplasmic streaming was induced at low Ca^{2+} concentrations but inhibited at high concentrations (Takagi and Nagai 1986).

Involvement of the Ca^{2+} flux across the plasma membrane in photodinesis was suggested. Takagi and Nagai (1988) evaluated the Ca^{2+} flux across the plasma membrane by monitoring the external Ca^{2+} concentration ($[Ca^{2+}]_{out}$) using mesophyll protoplasts. Red light induced an increase in $[Ca^{2+}]_{out}$ but far-red light led to a decrease. The effect of far-red light was inhibited by a Ca^{2+} channel inhibitor, nifedipine (Takagi and Nagai 1988). In the presence of La^{3+}, another Ca^{2+} channel inhibitor, illumination with far-red light did not induce stoppage of cytoplasmic streaming. Thus, Ca^{2+} influx through the Ca^{2+} channels of the plasma membrane is involved in inhibition of cytoplasmic streaming by far-red light. Vanadate inhibited both induction of cytoplasmic streaming and the increase in $[Ca^{2+}]_{out}$ by red light (Takagi and Nagai 1988). Red light may reduce cytoplasmic Ca^{2+} by accelerating its sequestering into the endoplasmic reticulum and/or by activating extrusion using the electrochemical potential gradient for H^+ across the plasma membrane.

Takagi et al. (1990) found that photosynthesis is involved both in induction of cytoplasmic streaming and in Ca^{2+} efflux regulated by the phytochrome system. The plasma membrane is equipped with an electrogenic proton pump energized by ATP. Because the proton pump is activated by light via photosynthesis, the plasma membrane hyperpolarizes upon illumination (Harada et al. 2002a, b). It has been suggested that hyperpolarization induces an increase in active Ca^{2+} efflux, although no H^+–Ca^{2+} antiporter is known in the plasma membrane of these cells. Plieth and Hansen (1992) reported that the velocity of primary cytoplasmic streaming of *N. flexilis* increased by approximately 15–30% upon illumination and that the increase did not occur in the presence of an inhibitor of photosynthesis. They argued for a possible decrease in the cytoplasmic Ca^{2+} concentration via the light-dependent uptake by the chloroplasts. An illumination-induced decrease in cytoplasmic Ca^{2+} in *Nitellopsis* sp. had been reported by Miller and Sanders (1987), although the change was small.

The cytoplasmic streaming in *V. gigantea* mesophyll cells is inhibited by cytochalasin B (Ishigami and Nagai 1980), and bundles of actin filaments are anchored at the track of cytoplasmic streaming (Takagi and Nagai 1983, Masuda et al. 1991; Ryu et al. 1995). Thus, the actin–myosin system is involved in motive force generation. The motile system is believed to be inhibited by Ca^{2+}. Movement of actin filaments on glass surfaces coated with an extract of *V. gigantea* was inhibited by Ca^{2+} (Takagi et al. 1995).

Positioning of organelles

Chloroplasts change their intracellular position in response to light conditions (Haupt 1982). Phytochrome and/or blue light-absorbing pigments such as phototropin are involved in this phenomenon (Yatsuhashi et al. 1985; Kadota et al. 2000; Sato et al. 2000; Kawai et al. 2003; Kasahara et al. 2004). The actin cytoskeleton participates in photo-orientation of chloroplasts (Dong et al. 1998; Sato et al. 2001). Since the architecture of the actin filaments changes extensively in response to light conditions (Dong et al. 1998), the involvement of any actin-binding protein is suggested. In moss protonemata, both actin and microtubule cytoskeletons are involved in photo-orientation of chloroplasts (Sato et al. 2000). Photomovement regulated by phytochrome is mediated by the microtubule system, whereas blue-light-regulated movement is mediated by both cytoskeletons (Sato et al. 2000). Physiological study has shown that influx of extracellular Ca^{2+} has no direct effect on the photo-orientation of chloroplasts (Tlalka and Gabrys 1993; Sato et al. 2001, 2003), whereas analysis using photoreceptor mutants suggested a light-dependent increase in Ca^{2+} influx. This discrepancy was discussed by Sato et al. (2003).

When cells of the diatom *Pleurosira laevis* are touched chloroplasts accumulate at the center of the cell (Makita and Shihira-Ishikawa 1997). This chloroplast translocation can also be induced by electrical stimulation or by an increase in KCl concentration. Either depletion of extracellular Ca^{2+} or addition of a Ca^{2+} channel inhibitor disturbed chloroplast accumulation. Application of a Ca^{2+} ionophore induced the response. It was suggested that Ca^{2+} influx due to mechanically

induced depolarization is involved in chloroplast accumulation (Makita and Shihira-Ishikawa 1997).

When fern protonemal cells are stimulated mechanically, chloroplasts leave the site of stimulation (Sato et al. 1999). It seems that actin filaments are involved, since the response was inhibited by cytochalasin but not by microtubule inhibitors. BDM also inhibited the response, suggesting the involvement of myosin. In moss protonemata, chloroplasts accumulate at the site of mechanical stimulation and the response is inhibited by inhibitors of microtubules but not by inhibitors of actin filaments (Sato et al. 2003). The involvement of Ca^{2+} influx from the external medium was suggested for both fern and moss (Sato et al. 2001, 2003).

When plants are attacked by fungus, cytoplasm accumulates at the site (Kunoh et al. 1985; Gross et al. 1993). Treatment of non-host plants with cytochalasin impaired this resistance, resulting in penetration by the fungus (Kobayashi et al. 1997a, b). Actin-based accumulation of cytoplasm is therefore responsible for the non-host resistance. Endoplasmic reticulum and Golgi bodies accumulate at the infection site (Takemoto et al. 2003). This suggests involvement of the myosin motor(s) responsible for transport of these organelles.

Actin cytoskeleton and expansion growth of cells

Cytoplasmic streaming is responsible for intracellular transport of organelles and molecules. It is also becoming evident that the actin cytoskeleton is involved in cell growth. *A. thaliana* has eight functional actin genes (Meagher et al. 1999). Replacement of one amino acid (arginine) with cystein in *ACT2* results in severe defects in initiation and elongation of root hair cells, elongation of root epidermal cells, and growth of the aerial portions of plants (Nishimura et al. 2003). The relationship between expansion growth and the actin–myosin system has been most extensively studied with pollen tubes.

The treatment of pollen tubes with cytochalasin B inhibits cytoplasmic streaming and tip growth (Franke et al 1972; Mascarenhas and Lafountain 1972). The cytoplasmic streaming is believed to be involved in the transport of the organelles and molecules necessary for tip growth. When pollen tubes are treated with caffeine, tip growth stops but not cytoplasmic streaming (Miler et al. 1996; Pierson et al. 1996; Lancelle et al. 1997). In addition to this transport, the mechanism of regulation of the actin cytoskeleton seems necessary for maintenance of the tip growth, as discussed below.

Brewbacker and Kwack (1963) reported that extracellular Ca^{2+} is necessary for germination of pollen grains and growth of pollen tubes. Using fluorescent dye, a tip-focused cytoplasmic Ca^{2+} gradient was discovered (Reiss and Nobling 1986; Nobling and Reiss 1987). When pollen tubes are growing actively, a clear zone lacking large organelles is formed at the tip region. Vigorous movement of organelles and large actin bundles is absent (Lancelle et al. 1987; Tiwari and Polito 1988; Lancelle and Hepler 1992; Miller et al. 1996,). When tip growth was inhibited by caffeine treatment, the clear zone becomes narrow and large organelles invade the space of the original clear zone. Thick actin bundles extend to the tip region (Miller et al. 1996; Pierson et al 1996; Lancelle et al. 1997). Microinjection of a Ca^{2+} chelator also has the same effect (Miller et al. 1992; Pierson et al. 1994). Thus, the presence of a clear zone maintained by the tip-focused Ca^{2+} gradient is necessary for tip growth.

Biochemical study of pollen tubes has shed light on the mechanism of formation of the clear zone. Two isoforms of villin were isolated from lily pollen tubes (Nakayasu et al. 1998; Yokota et al. 1998; Vidali et al. 1999). The activity of villins to bundle actin filaments is inhibited in the presence of both Ca^{2+} and calmodulin (Yokota et al. 2000, 2003). In the presence of Ca^{2+}, villins also show activities of capping and fragmentation of the actin filaments (Yokota et al. 2005).

Huang et al. (2004) and Fan et al. (2004) reported that actin-severing proteins which belong to the gelsolin superfamily are also involved in Ca^{2+}-dependent organization of actin filaments in pollen tubes. Actin depolymerizing factor (Allwood et al. 2002; Chen et al. 2002, 2003), ROP GTPase (Gu et al. 2003), and profilin (McKenna et al. 2004) also seem to participate in the regulation of the actin dynamics in pollen tubes.

The sliding activity of myosin XI is inhibited by Ca^{2+} (Yokota et al. 1999a). Thus, the high Ca^{2+} concentrations of may suppress active movement of organelles in the tip region.

Perspectives

Two sliding theories for the mechanism of cell motility, muscle contraction (Huxley and Niedergerke 1954; Huxley and Hanson 1954) and cytoplasmic streaming (Kamiya and Kuroda 1956), were proposed within two years of each other. In both cases, the sliding occurs between myosin and actin filaments. Due to the different C-terminus functions of myosins, completely different phenomena are expressed. Since myosin II forms bipolar aggregates, sliding causes muscle

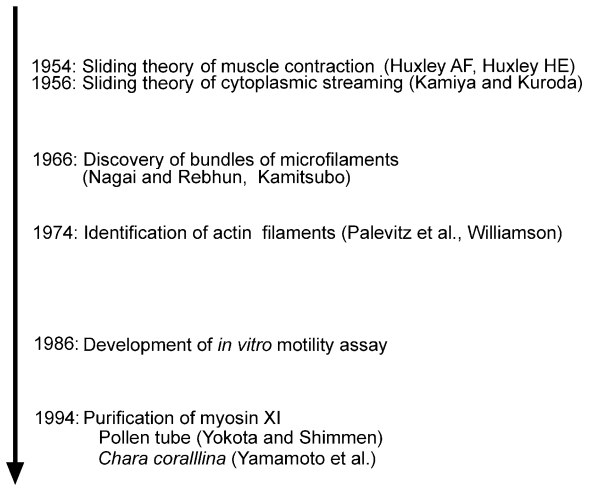

Fig. 4 Steps of discovery of the actin and myosin responsible for cytoplasmic streaming in plant cells

contraction. Since myosin XI binds to organelles, the motive force of the cytoplasmic streaming is generated.

Studies on the main components necessary for generation of the motive force of cytoplasmic streaming, actin filaments and myosin XI, have progressed in approximately ten-year steps (Fig. 4). A decade elapsed between the proposal of the sliding theory and the finding of the bundles of microfilaments, and then approximately another 10 (8) years for the microfilaments to be identified as actin filaments. It took a further 20 (10×2) years for purification of myosin XI.

Various roles of the actin–myosin system in the expression of plant cell functions are becoming evident. Further developments of molecular biology are expected to promote understanding of the plant actin–myosin system in collaboration with physiological, morphological, and biochemical approaches.

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