Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall

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Summary

Myosins are a large superfamily of motor proteins which, in association with actin, are involved in intracellular motile processes. In addition to the conventional myosins involved in muscle contractility, there is, in animal cells, a wide range of unconventional myosins implicated in membrane-associated processes, such as vesicle transport and membrane dynamics. In plant cells, however, very little is known about myosins. We have raised an antibody to the recombinant tail region of Arabidopsis thaliana myosin 1 (a class VIII myosin) and used it in immunofluorescence and EM studies on root cells from cress and maize. The plant myosin VIII is found to be concentrated at newly formed cross walls at the stage in which the phragmoplast cytoskeleton has depolymerized and the new cell plate is beginning to mature. These walls are rich in plasmodesmata and we show that they are the regions where the longitudinal actin cables appear to attach. Myosin VIII appears to be localized in these plasmodesmata and we suggest that this protein is involved in maturation of the cell plate and the re-establishment of cytoplasmic actin cables at sites of intercellular communication.

Introduction

The myosins are a large superfamily of molecular motors which generate movement and mechanical force in ATP-dependent interactions with actin filaments. On the basis of their conserved head or motor domain sequences, i.e. the highly conserved region containing the ATPase and actin binding sites, the myosins can be divided into at least 14 classes (designated I to XIV) (Cope et al., 1996; Mermall et al., 1998). In addition, each class of myosin contains tail domains which are characteristic for each myosin and are believed to be responsible for the specific subcellular localization and function of these motors.

In unicellular systems and mammalian cells the involvement of the conventional two-headed, filament-forming myosins (myosin IIs) is well established in muscular contraction and cytoplasmic contractile events such as cytokinesis. In addition, the so-called unconventional myosins of class I, V and VI are now relatively well described; several of them have recently been shown to be involved in various actin-based and membrane-associated functions, which include vesicle transport, cytokinesis and such specialized functions as auditory perception (for recent reviews see Bährler, 1996; Fath and Burgess, 1994; Hammer, 1994; Hasson and Moo seker, 1995; Mermall et al., 1998; Mooseker and Cheney, 1995; Titus, 1993).

In comparison to the mammalian and protozoan myosins, little is known about these motors in higher plants (for review see Asada and Collings, 1997; Kendrick-Jones and Reichelt, 1999). The most prominent intracellular motile event depending on the actomyosin cytoskeleton in plant cells is cytoplasmic streaming, i.e. the movement of vesicles and organelles in Chara internodal cells and pollen tubes (for review see Kuroda, 1990; McCurdy and Williamson, 1991; Williamson, 1993). In Chara cells cytoplasmic streaming occurs at rates of up to 60 μm sec⁻¹ (about an order of magnitude faster than the speed of the fastest muscle) (Kachar and Reese, 1988). A recently isolated myosin protein from Chara (Higashi-Fujime et al., 1995; Yamamoto et al., 1995) moves muscle F-actin in vitro motility assays at a similar velocity, strongly suggesting that the isolated protein is the motor responsible for cytoplasmic streaming. In Lily pollen tubes (Miller et al., 1995; Tang et al., 1989), and in various tissues from several plants (La Claire, 1991; Qiao et al., 1989; Turkina et al., 1987; Vahey et al., 1982; Yokota et al., 1995), biochemical and immunofluorescence studies using heterologous myosin antibodies have detected myosin-like proteins. Most of these reports demonstrated spot-like anti-myosin labelling throughout the cytoplasm and along actin filaments, and this was interpreted as
showing the association of putative myosins with various vesicles and organelles (Grolig et al., 1988; Grolig et al., 1996; Heslop-Harrison and Heslop-Harrison, 1989; Miller et al., 1995). A more recent study has shown heterologous anti-myosin staining associated with plasmodesmata in Chara cells (Radford and White, 1998). However, none of these putative myosins proteins has so far been cloned and sequenced to confirm their myosin identity.

The first myosin gene to be cloned and sequenced from a plant, Arabidopsis thaliana myosin 1 (ATM1) (Knight and Kendrick-Jones, 1993), was placed by phylogenetic analysis of its motor domain sequence into a new class of myosins (class VIII). Since then a further 10 plant myosin genes (from Arabidopsis, Helianthus, Acetabularia and Chlamydomonas) have been cloned and sequenced (Kinkema and Schiefelbein, 1994; Kinkema et al., 1994; La Claire et al., 1995; Vugrek and Menzel, personal communication) and, on the basis of their conserved motor domain sequences, have been placed into three new classes (classes VIII, XI and XIII) (Cope et al., 1996) by phylogenetic analysis. Further analysis of their highly divergent C-terminal tail sequences confirms this classification.

None of the plant myosins identified thus far belong to class I or II. The plant myosins in class XI are closely related to the animal myosin Vs but are sufficiently different to be grouped into a separate class (Cope et al., 1996). Furthermore, PCR screens and the Arabidopsis genome sequencing project indicate the existence of additional myosins in classes VIII, XI and XIII (Kinkema et al., 1994; La Claire et al., 1995; Moepps et al., 1993; Plazinski et al., 1997; Vugrek and Menzel, personal communication).

The important point to emerge from this analysis is that all the myosin genes identified from plants belong to unique classes which do not contain animal or protozoan myosins. The motile processes in higher plants and animals may be specifically different and, in order to investigate the plant-specific localization and function, we generated an antibody against a recombinant tail region of myosin ATM1. The immunolabelling patterns in root cells from Lepidium sativum (cress) and Zea mays (maize) indicate that this myosin VIII is predominantly localized at the cell periphery where it is preferentially associated with those plasma membrane regions involved in the assembly of new cell walls. The immunogold and immunofluorescence images reveal that this myosin is also associated with cell-to-cell contact areas in root cells and appears to accumulate specifically in plasmodesmata. These results suggest the intriguing possibility that in plant cells actomyosin-based forces are involved in the selective ‘gating’ of plasmodesmata.

**Results**

**Immunological studies of myosin VIII in root tissue preparations**

To raise antibodies specifically against the Arabidopsis thaliana myosin (ATM1), a class VIII plant myosin, we expressed and purified its tail region and used it as the immunogen. This tail region appears to be unique and consists of a short stretch of predicted α-helical coiled-coil (amino acids 950–1019 containing about 10 heptad repeats) and a unique C-terminal region (residues 1020–1166) (Knight and Kendrick-Jones, 1993) which has no apparent homologies or recognisable motifs. The only noticeable features are two clusters of serine residues and a cluster of basic residues at its very C-terminus. For expression, a vector of the pGEX series was modified so that fusion proteins with GST (Glutathione-S-transferase) on their N-termini and a stretch of six histidine residues on their C-termini could be expressed. This vector is especially useful when, as in this case, proteolytic degradation of the expressed protein is a problem. Using the modified vector with two affinity labels, intact fusion protein could be prepared for antibody production.

For our immunological studies, we used root tissue from Arabidopsis thaliana, cress (Lepidium sativum) and maize (Zea mays) and coleoptile tissue from maize for Western blots. Since cress and Arabidopsis belong to the same dicot family (Brassicaceae), antibodies raised against the Arabidopsis thaliana myosin 1 (ATM1) were expected to cross-react with a myosin of the same class in cress and this appears to be the case. We also found that we could obtain the same immunofluorescence pattern in maize roots.

To check the specificity of the affinity-purified polyclonal anti-ATM1 antibody, immunoblot analyses were carried out on subcellular fractions from cress root and maize coleoptiles (Figure 1). In both cress roots (Figure 1A,a) and maize coleoptiles (Figure 1B,b), the antibody cross-reacts with a 130 kDa band in the microsomal membrane fraction (Figure 1). This subcellular distribution indicates that the intact 130 kDa myosin VIII is associated with the membrane fraction, reminiscent of the membrane localization of the myr 4 myosin in rat brain fractions (Bähler et al., 1994). In our experience, many of the unconventional myosins in animal tissues and tissue culture cells are preferentially bound to cytoskeletal-membrane fractions. In cress roots there is also a faint cross-reacting band at 170 kDa (Figure 1a) which may be the product of one of the class XI myosin genes recently identified by Kinkema and Schiefelbein (1994). When protein extracts were rapidly prepared from cress roots in the presence of a wide spectrum of protease inhibitors and MgATP and subjected to immunoprecipitation with the anti-ATM1 antibody, a single band of approximately 130 kDa was detected on Western blots.
Figure 1c. The size of the immunoprecipitated protein is in good agreement with the predicted molecular weight of the ATM1 protein (131.2 kDa) (Knight and Kendrick-Jones, 1993). There was no corresponding Coomassie-stained band on the SDS-PAGE gel (Figure 1C) indicating that the amount of myosin VIII in these tissues is likely to be very low. Purified myosin VIII tail fusion protein when mixed with anti-myosin antibody specifically blocked the Western blot and immunofluorescent staining patterns.

Immunolocalization of myosin VIII along the transverse cell walls in root tissue

For the immunolocalization studies, we used root tissue from Arabidopsis, cress and maize seedlings. Root tissues were preferred because they are more convenient to handle and their simple architecture allows one to consistently identify the various cell types undergoing different developmental programmes. Virtually identical antibody labelling patterns were obtained with root tissues from all three plants (images from Arabidopsis are not shown). Cress and maize roots were preferred: cress because it is closely related to Arabidopsis and its roots are more robust, and maize because its cytoskeleton has been comprehensively studied (e.g. Baluska et al., 1992; Blancaflor and Hasenstein, 1995).

The anti-ATM1 antibody strongly labelled the peripheral region underlying the transverse cell walls in the roots of all three plants (Figures 2b,d and 3b). In contrast and acting as an internal control, the longitudinal walls in these cells exhibited rather punctate myosin VIII labelling (Figure 2d,h). The labelling was most prominent in the region containing meristematic and early post-mitotic cells which were undergoing cell division. The strongest labelling was found along newly formed cell walls which were in the process of maturation.

Controls using either pre-immune serum or secondary antibody alone or antibody to vertebrate non-muscle myosin II (Drenckhahn et al., 1983) did not show any staining in root cells (data not shown).

Myosin VIII localisation during mitosis

The distribution of myosin VIII, actin and microtubules during stages in the cell cycle in root meristems in higher plants are shown in Figures 2 and 3 and are summarised in the schematic drawings in Figure 6. At the onset of mitosis (prophase), actin is localised as dense irregular networks surrounding the enlarged, round nucleus (Figures 2a, 3a and 6a). At this stage, myosin VIII labelling is concentrated along the transverse cell walls and is sparsely distributed along the longitudinal cell walls (Figure 2b). At the beginning of metaphase, when the nuclear envelope disintegrates, actin staining is concentrated along the two opposing transverse cell walls with a few actin filaments reaching towards the centre of the cell (Figures 2g and 6b). Myosin VIII labelling is more intense than in prophase and is concentrated at the opposing transverse cell walls, co-localizing with the actin filaments (Figures 2h and 6b).

The different stages in the cell cycle can be readily assigned by following the distribution of microtubules and mitotic chromosomes. At metaphase, when the chromosomes are aligned midway between the spindle poles, dense bundles of kinetochore microtubules connect the chromosomes to the poles (see SP in Figures 3g and 6d). During telophase, in the early phragmoplast (P) region where the new cell wall forms between the separating daughter nuclei, intense anti-tubulin staining is seen as a broad phragmoplast band in the centre of the newly forming cell plate (Figures 3d and 6e). In late cytokinesis, the phragmoplast P* forms a ring-like structure surrounding the newly developing cell plate (Figures 3d,g and 6f). Myosin VIII staining does not occur in the callose-rich cell plate region in the early (P) or in the developing late phragmoplast (P*) (Figures 3c,f and 6f) but is confined entirely to the opposing transverse cell walls which are strongly stained (Figure 3c,f,h). The DAPI-stained chroma-
tin in these cell stages is still slightly condensed (Figure 3e,i), and the shape and small size of the nuclei reveal that these cells are in a late phase of cell division. Later, in G1-phase (G in Figure 3), shortly after disintegration of the phragmoplast microtubules (Figure 3d,g), extensive labelling of the myosin VIII was detected in this area (Figures 3c,f,h and 6g). In these cells the chromatin in the nuclei is uncondensed (Figure 3e,i), and the phragmoplast microtubules have become integrated into the cortical microtubule network (Figures 3g and 6h). At this late G1 stage, anti-ATM1 staining is thus very strongly concentrated along or in the newly formed cell wall (Figure 3b,f,h) and the actin filaments are arranged in a regular network reaching from the cell walls to the nucleus (Figures 2c,e and 6c). It should be noted that during all these stages the transverse cell walls are always highly labelled with anti-ATM1 antibody whereas the longitudinal walls show only punctate labelling (Figures 2b,d,h and 3b).

In interphase cells, after completion of cell division, the transverse cell walls are stained with anti-ATM1 antibody, but less brightly than during mitosis (Figure 2d,f). Actin staining of the same region shows a very prominent F-actin array, with characteristiclly arranged actin filament bundles (Figures 2c,e and 6i). The actin co-localizes with the myosin VIII labelling along the transverse cell walls and extends into the cell centre where the bundles appear to separate into several finer filaments surrounding the nucleus. These finer central actin bundles are not labelled with myosin VIII.

**Association of myosin VIII with the plasma membrane and plasmodesmata**

1. Confocal microscopy. Optical sections through cress root tips labelled with the anti-ATM1 antibody confirmed that the myosin VIII was localized along the whole transverse cell wall, whereas the longitudinal walls show only punctate labelling. An overlay of a phase-contrast image and the fluorescent myosin-staining is shown in pseudo-colour (Figure 4a). The myosin VIII labelling pattern is densely punctate, with the highest density of label in new cell walls in early G1, shortly after phragmoplast disintegration. An overlay of optical sections (Figure 4b) reveals that the punctate labelling pattern passed through the whole cell wall area. Along the longitudinal cell walls a less dense distribution of myosin VIII in distinct patches can also be observed. In Figure 4(c), the myosin VIII labelling in G1-phase cells is shown at a higher
magnification, revealing intense myosin VIII labelling of the plasma membrane associated with the newly formed cell wall.

2. Immunogold electron microscopy. To confirm these immunofluorescence images, we prepared ultra-thin frozen sections of cress roots and subjected them to indirect immunogold labelling with our anti-ATM1 antibody. In the electron microscope, labelling was detected along the newly formed cell wall between two cells (arrows indicate the 10 nm immunogold particles in Figure 5a,b). This cell wall shows the features of a young cell wall after the disintegration of the phragmoplast and before it loses its ‘wavy’ appearance, characteristic of the final stages in the maturation of the cell wall. The anti-ATM1/immunogold labelling (10 nm particles) occurred in association with the plasma membrane and at thin membranous structures going through the cell wall which are thought to be plasmodesmata. Other regions of the sections were virtually free of any immunogold labelling.

For a more detailed localisation of the myosin in the cell walls, we used ultra-thin sections of maize roots embedded in LR White Resin which preserves the membrane ultrastructure better than cryo-fixation. Transverse as well as longitudinal cell walls possessing primary and secondary plasmodesmata showed characteristic anti-ATM1/immunogold labelling (Figure 5c,d,e). In young transverse cell walls, plasmodesmata were seen decorated with strings of gold particles (Figure 5c) and in longitudinal cell walls, where secondary plasmodesmata were mostly arranged in large pit fields, the immunogold particles were especially enriched in this area (Figure 5d,e). The cytoplasm and other cell structures were very rarely labelled when compared with the pit fields.

Discussion
We have performed the first cytological characterization of an unconventional myosin in higher plants using an
antibody specifically raised against a recombinant tail region of the first plant myosin gene to be cloned and sequenced. This gene (ATM1) was isolated from a dicot plant Arabidopsis thaliana (Knight and Kendrick-Jones, 1993). The predicted molecular weight of ATM1 is 131 kDa, and it contains a motor domain (head), four IQ-motifs predicted to bind calmodulin or light chains and a tail domain with a predicted short coiled-coil region which implies its dimerisation into a double-headed molecule. The whole tail region of this myosin was expressed and used as an immunogen to generate specific antibodies. Immunoblot analysis using these anti-ATM1 antibodies identified an approximately 130 kDa band which is highly enriched in the membrane fraction in cress and maize tissues. These results suggest that the myosin VIII is membrane-bound, possibly by its C-terminal globular region. In this connection it is interesting that the animal myosin Is and myosin Vs are known to be bound to membranes (Mooseker and Cheney, 1995).

Immunofluorescence and immunoelectron microscopy of cress and maize root tissues using the anti-ATM1 antibody showed that the myosin VIII is concentrated along the plasma membranes at transverse cell walls both in meristematic cells and in the post-mitotically growing root cells. Cells in G1 phase are extensively stained along their young transverse cell walls, whereas there is only punctate staining along longitudinal cell walls. The immunolocalization patterns show that myosin VIII is concentrated along the transverse walls where F-actin is attached perpendicularly. This suggests that myosin VIII may have some role in the formation of new cell walls as it has a less dense distribution along established side walls. One key feature of the new end walls is that they are a specialized zone of concentrated cell–cell contact where plasmodesmata are formed at cell division. It is significant, therefore, that immunogold studies show myosin to be located at these intercellular plant cell junctions.

In previous studies, myosin-like proteins from plants have been characterized either with cross-reacting antibodies or by stimulation of their ATPase activity by F-actin (for review see Asada and Collings, 1997; Kendrick-Jones and Reichelt, 1999). Such studies have revealed a variety of proteins, perhaps reflecting the diverse classes of myosins. One 240 kDa protein has been isolated from Chara that moves muscle F-actin at 60 μm sec⁻¹ in in vitro motility assays which strongly suggests that this protein is the motor responsible for cytoplasmic streaming. This myosin when viewed in the electron microscope after rotary shadowing (Yamamoto et al., 1995) has similar characteristics to a myosin V, i.e. it is a dimer of two 210–220 kDa heavy chains with pear-shaped heads (motor domains) and a slightly curled globular tail about 80 nm long. Thus far it has not been possible to clone and sequence the gene for this Chara myosin. The predicted molecular weights of
class VIII myosins is about 130 kDa and for class XI myosins about 170 kDa. In higher plants, a 170 kDa myosin has been biochemically isolated from lily pollen tubes (Yokota and Shimmen, 1994) and an antibody to this protein recognize 170 kDa polypeptides in tobacco, *Tradescantia* and *Arabidopsis* tissue (Yokota et al., 1995). When heterologous anti-myosin antibodies have been used to stain plant cells, they have mostly produced punctate staining patterns throughout the cell which is indicative of the labelling of organelles and vesicles involved in membrane transport (Grolig et al., 1988; Miller et al., 1995; Qiao et al., 1989). It can be concluded that homologues of the myosin superfamily exist in higher plants and are involved in a variety of membrane associated processes. However, it is clear that the anti-myosin VIII localization reported here has distinct differences from these previous studies. One of the striking features of myosin VIII localization is that it becomes concentrated in newly deposited cross-walls in contrast to its sparse occurrence along side walls. This suggests that it may be involved, not so much in the process of vesicle transport and fusion which bring cell plate precursors to the mid-line of the phragmoplast, but in those processes of cell plate maturation. Indeed, myosin VIII labelling is found most strongly when the actin and microtubules of the phragmoplast have depolymerized, and it is important to examine the stage-specific processes in which myosin VIII may be implicated.

Several studies show that cell wall formation occurs in two separate stages. For instance, caffeine is known not to effect the formation of the phragmoplast cytoskeleton nor the actual deposition of the central cell plate, but the later stage of fusion with the mother wall and the conversion of the callose-rich plate into a less flexible wall (Hepler and Bonsignore, 1990; Valster and Hepler, 1997). Mineyuki and Gunning (1990) have reviewed these later stages of cell division and concluded that insertion and maturation factors interact with the cell plate or pass into it from the cortical division site. Callose (a major component of the cell plate) is removed from cell plates soon after they attach (Northcote, 1989) and pectic polysaccharides are inserted (Moore and Staehelin, 1988; Samuels et al., 1995).
The appearance of myosin VIII coincides therefore with the onset of these wall-consolidating processes. The protein could bring the islands of membrane plate material together or it could trigger the exocytosis of new cell wall material. High concentrations of calcium–calmodulin are found near the phragmoplast/cell plate (Hepler, 1994) and

Figure 6. Schematic drawings of the distribution of myosin VIII, actin filaments and microtubules in cells of the meristem and transition growth zone in the root apex.

Cell walls and the underlying plasma membrane are represented as one grey line. The drawings represent the distribution of the cytoskeletal elements in the median level of the cells, only in late G1/S-phase are cortical microtubules shown (as indicated). (a) In prophase, actin filaments are distributed as dense irregular networks surrounding the enlarged round nucleus (Figure 2a). Myosin VIII labelling is concentrated along the transverse cell walls and sparsely distributed along the longitudinal cell walls (Figure 2b,d). (b) In metaphase, the nuclear envelope disintegrates and the chromosomes are arranged along the equatorial plane. The F-actin at this stage forms a dense network of short filaments on the two opposing transverse cell walls with a few actin filaments reaching towards the cell centre, but not into the equatorial plane (Figure 2g). Myosin VIII labelling is more dense than in prophase, concentrated at the opposing cell walls, co-localizing with actin (Figure 2h). (c) In G1-phase, the newly formed plasma membrane and cell wall separates the daughter cells. Actin filaments are arranged in a regular network reaching from the cell walls to the nucleus (Figure 2e). The highest density of myosin VIII labelling is located along the newly assembled cell wall (Figure 2d). The transverse cell walls are also strongly labelled with ATM1 antibody whereas the longitudinal walls show only weak punctate labelling. The different cell cycle stages can be identified by the distribution of microtubules and the chromosomes. (d) At metaphase, when the chromosomes are aligned midway between the spindle poles, dense bundles of microtubules connect the chromosomes to the pole (Figure 3g). (e) After separation of the chromosomes into daughter nuclei in late telophase, microtubules of the early phragmoplast (P-MTs) are concentrated between the separating nuclei (Figure 3d). At this stage, numerous Golgi-derived vesicles accumulate at the equatorial plate to initiate cell plate formation. (f) During late telophase/G1-phase, the late phragmoplast microtubules (P*-MTs) are concentrated as a ringlike-structure surrounding the leading edges of the assembling cell plate (Figure 3d,g). Additional vesicles fuse with the phragmoplast, extending it outwards and eventually the phragmoplast fuses with the plasma membrane, and the two cells separate. In (d–f) no myosin VIII staining was observed in the mitotic spindle during the separation of the chromosomes or in the early or late phragmoplast (Figure 3c,f). However, the opposing transverse cells walls are strongly stained with myosin VIII during these stages in the cell cycle, co-localizing with actin filaments during metaphase. (g) In early G1-cells, after phragmoplast disintegration and cell plate assembly, the ATM1 labelling becomes concentrated along the newly formed cell wall which separates the daughter cells (Figure 3b,c). (h) Later in G1-phase, when the nuclear membranes have completely reformed around each daughter nucleus and the nucleoli are visible (Figure 3e,i), the microtubules and the actin are arranged as dense networks interconnecting the centrally located nucleus with the cell periphery. At this stage, the highest density of ATM1 labelling is located along the newly assembled cell wall (Figure 3c). (i) During interphase, when the root cells elongate, the actin filaments are extending from perinuclear sites towards the opposite transverse walls of the cell where they co-localize with the myosin VIII (only two actin bundles in the median level are shown) (Figure 2c,d). These actin filaments may form a transport pathway for Golgi-derived vesicles carrying new cell wall components.
it is possible that changes in the calcium levels at the transition between plate deposition/maturation affect the localisation and activity of myosin VIII. In Chara, a polyclonal antibody raised against smooth and skeletal myosin has been shown to label plasmodesmata (Radford and White, 1998) – a localisation pattern which agrees with our previous results (Reichelt et al., 1997) and this paper.

In the early post-mitotic root cells of cress and maize the transverse walls are intensely labelled with the anti-myosin VIII and actin antibodies. This myosin antibody does not decorate F-actin cables involved in cytoplasmic streaming but it is interesting that myosin VIII is concentrated along those walls to which the longitudinal actin cables are directed and attached. The cytoplasmic actin cables largely depolymerise in cell division but streaming is restored along the interphase array during early postcytokinesis. Another possible role for myosin VIII (which is detected in the membrane fraction) is that it may be part of the system for anchoring or directing the cytoplasmic F-actin cables. Since F-actin goes to (or through) plasmodesmata (White et al., 1994), it has been suggested to be involved in the intercellular movement of certain viruses (McLean et al., 1995), proteins and mRNA (Lucas et al., 1996). Such particle movement is most likely to be an exaggerated form of normal directed cell transport. It is interesting therefore that the density of plasmodesmata is far greater in the transverse end walls than in the longitudinal side walls (Juniper and Barlow, 1969). The movement of virus particles (McLean et al., 1995; Waigmann and Zambryski, 1995; Zambryski, 1995), and the fact that cytochalasin D enlarges the plasmodesmatal neck (White et al., 1994), indicates that there is a gating mechanism which controls the size of the pore and that this is likely to be under contractile regulation (Ding et al., 1996; Overall et al., 1982; White et al., 1994). The present study has shown that myosin VIII is localized at plasmodesmata and, particularly since it is an actin-interacting protein, is a strong candidate for being part of any intercellular gating complex.

**Experimental procedures**

**Materials**

Analytical grade reagents were obtained from BDH Chemicals Ltd (Poole, UK), Bethesda Research Laboratories Inc. (Gaithersburg, MD, USA) and Sigma Chemical Co. Ltd (Poole, UK). Enzymes supplied by New England BioLabs (Hertfordshire, UK) and Promega Corporation (Madison, WI, USA) were used according to the manufacturer’s standard assay conditions. Biochemicals were supplied by Amersham International (Amersham, UK). Oligonucleotides were synthesized on an Applied Biosystems 390B automated synthesizer (Applied Biosystems Inc., Foster City, CA, USA) by Terry Smith (Laboratory of Molecular Biology, Cambridge, UK).

**Expression of recombinant ATM1 and antibody preparation**

The vector pGEX-H6 used for the expression of the tail region of Arabidopsis thaliana myosin VIII (ATM1) is based on pGEXAT-3 (Pharmacia) with the addition of a C-terminal string of six histidine residues. This allows the intact expressed protein to be purified using two specific affinity tags by the so-called ‘affinity-sandwich’ protocol (Binder et al., 1994). The vector was constructed by inserting a double-stranded oligonucleotide consisting of: 5’-GGC CGC CAT CAC CAT CAC CAT CAC CAT-3’ and 5’-GGC CAT GGT GAT GAT GAT GGT GGC-3’ into the NotI site of pGEXAT-3. This oligonucleotide introduces the 6 His (H) sequence C-terminal to a regenerated NotI site such that if the insert is correctly engineered this His-tag should remain in frame with both upstream sequences and the downstream stop codon.

The ATM1 insert was produced by PCR using the intact ATM1 cDNA clone (3.851Kb) (Knight and Kendrick-Jones, 1993) as template and the following oligonucleotides: 5’-CGA ATT CCC GGG TCG GAG GTG CCA AGA CAA ATG AGT TAG GTG AG-3’ and 5’-GCC ACG ATG CCG CCG GTA TAC CTG GTG CTA TTT CTC CTT CCC CAC CA-3’ as primers which also introduce NotI and XmnI restriction sites (underlined). The PCR product (2952–3632 in the ATM1 cDNA) containing the whole tail-region of ATM1 including the coiled-coil and the C-terminal globular regions (amino acid residues 938–1166) was first blunt-end cloned into puc18, digested with HindIII and transformed into E. coli JM101 cells. Positive clones were recognized by blue/white selection and PCR screening and were sequenced. Clones which contained the correct inserts were digested with NotI and XmnI and ligated into the dephosphorylated expression vector pGEX-H6. The ATM1-pGEX-H6 construct was expressed in E. coli BL21 (DE3) cells (Studier and Moffat, 1986). Freshly transformed colonies were inoculated into 2XTY medium containing 100 mg ml⁻¹ ampicillin, grown overnight at 37°C and then induced with 0.6 mM IPTG and grown for a further 3 h at 37°C. The cells were harvested by centrifugation at 5000 g and resuspended in PBS containing 1% TritonX-100 and a battery of protease inhibitors (see section on immunoprecipitation and Western blot analysis). The cells were lysed in a French Press and the supernatant collected by centrifugation at 12 000 g. The ATM1 was expressed as a soluble fusion protein, GST-ATM1-H6, with a molecular weight of 54 kDa. The supernatant was filtered through a 0.45 µm filter and applied to a Glutathione(GT)-Sepharose 4B column and purified according to the manufacturer’s instructions (Pharmacia P-L Biochemicals Inc.). After application of the expressed protein, the GT-Sepharose column was washed with at least four volumes (volume of supernatant applied) of PBS containing 1% Triton X-100 and a battery of protease inhibitors (see section on immunoprecipitation and Western blot analysis). The cells were further purified by SDS-PAGE on a 10% acrylamide mini-gel and the major band cut out and electroeluted. Three 100 µg aliquots of this fusion protein were used for antibody production in rabbits, as described by Harlow and Lane (1988). The resulting polyclonal serum was affinity-purified for the localization and Western blotting studies in two steps; first using a glutathione-S-transferase (GST)-agarose affinity column to remove any anti-GST antibodies and then using the recombinant ATM1 protein on immunoblots to purify the anti-ATM1 antibodies. For these immunoblots the recombinant...
protein purified on a G7-Sepharose column was separated on an SDS-Page gel and transferred to nitrocellulose. The nitrocellulose blot was stained with Ponceau red and the strips containing the recombinant ATM1 were cut, blocked with 5% non-fat dry milk in TBS (Tris buffered saline) for 1 h and then incubated with the undiluted polyclonal serum overnight at 4°C on a rotating wheel. The nitrocellulose strips were washed repeatedly with TBS containing 0.05% Tween 20 and the bound ATM1-antibodies eluted by vortexing the strips for 30 sec in 0.2 M Glycine, pH 2.2, and then quickly neutralizing with 1 M Tris±HCl, pH 7.6. The specificity of these purified antibodies was checked on blots; they strongly cross-reacted against GST-ATM1 fusion protein and thrombin cleaved 28 kDa fragment (see below) but gave no reactivity against GST even at low dilution. These antibodies weakly cross-react with the tail regions of purified chicken skeletal and brush border cytoplasmic myosin IIs. To detect the ATM1 protein in cells by immunoprecipitation, fresh Western blot analysis

**Immunoprecipitation, subcellular fractionation and Western blot analysis**

To detect the ATM1 protein in cells by immunoprecipitation, fresh 2-day-old cress roots were homogenized in an ice-cold solution containing 0.2 M KCl, 15 mM Tris–HCl, pH 7.2, 1 mM EDTA, 5 mM MgSO4 and 5 mM EGTA, pH 6.9 for 1 h at room temperature. To prepare fractions containing the membrane bound proteins, plant material (cell cultures from Arabidopsis, cress roots and maize coleoptiles) was homogenized (10 min in a mortar) in an ice-cold solution of 250 mM Tris–HCl (pH 8.0), 25 mM EDTA, 330 mM sucrose, 5 mM DTT, 5 mM ascorbic acid and 1 mM phenyl-methylsulphonyl fluoride (PMSF) followed by filtration through one layer of nylon mesh. All subsequent steps were carried out at 4°C. The post-mitochondrial supernatant (15 min, 4000 g; Biofuge 22R-Heraeus, Offenbach, Germany) was centrifuged for 50 min at 13,600 g (Biofuge 22R-Heraeus). The resulting pellet was used as the membrane bound protein fraction. For Bradford analysis the latter sample was resuspended in 200 μl of a solution containing 10 mM MES/bis-tris-phosphate (BTP) pH 7.5, 5 mM EDTA and 20% glycerol.

SDS-PAGE was performed on these samples diluted in sample buffer containing 3% SDS, 6% sucrose, 100 mM EDTA, 60 mM Tris–HCl, pH 6.8, and 0.001% bromphenol blue. The samples were heated to 80°C for 10 min, centrifuged and the supernatant loaded onto 8% gels. After protein separation, the gels were stained with Coomassie blue or electrophoretically transferred to nitrocellulose using a transblot cell (Bio-Rad, München, Germany). For immunostaining (at room temperature) the nitrocellulose membrane was pre-incubated with 4% BSA in TBS for 30 min, washed for 30 min (three times for 10 min) in TTBS (TBS buffer containing 0.05% Tween-20) and incubated with anti-ATM1 antibody (diluted 1: 100 in TTBS; control in TTBS). After washing in TTBS the membrane was incubated for 1 h in TTBS containing the secondary antibody (goat anti-rabbit IgG, whole molecule) conjugated to alkaline phosphatase (Sigma A-9919; dilution 1: 100 000). Washing in TTBS was followed by alkaline phosphatase detection using the fast-red staining protocol described by Druguet and Peps (1977).

**Immunofluorescence and immunogold localization of ATM1**

The specimens for immunofluorescence microscopy were prepared by the method of Baluska et al. (1992). Briefly, 2-day-old cress and maize roots were cut and fixed in 4% formaldehyde in MTSB (Microtubule Stabilizing Buffer) containing 50 mM PIPES, 5 mM MgSO4 and 5 mM EGTA, pH 6.9 for 1 h at room temperature. The roots were dehydrated in a graded ethanol series (30% to 97% ethanol in PBS) (PBS contained 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, 3 mM NaN3, pH 7.3) then incubated in wash ethanol (1: 2; 1: 1 and 2: 1 steps) and finally infiltrated in 100% Steedman’s wax (PEG-400 diestearate and 1-hexadecanol, 9: 1 (w/v)) at 37°C. The infiltrated roots were then embedded by allowing the wax to polymerize at room temperature. Longitudinal sections 4 μm thick were cut with a microtome (Jung, Heidelberg, Germany) and mounted on slides coated with glycerol-albumen (Serva). The sections were dewaxed in 100% ethanol, rehydrated in an ethanol series (97% to 30% ethanol in PBS), washed with PBS and then finally with MTSB. If sections were subjected to immunostaining with anti-actin antibody, incubation in methanol at -20°C for 10 min was required after

the ethanol series (Vitha et al., 1997). After methanol, the sections were washed with PBS and then MTBS. For immunostaining the sections were incubated with the affinity-purified anti-ATM1 antibody (diluted 1:200 in PBS with 0.1% BSA) or monoclonal anti-actin (ICN, anti-actin, clone C4, cat. no. 69–100) or anti-actin antibody (Amersham, N356) (both diluted 1:200 in PBS-BSA) for 60 min at room temperature. After washing with MTBS, the sections were incubated with fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG raised in goat (Sigma Chemical Co, St. Louis, MO, USA, F-6005) or rhodamine-conjugated anti-mouse IgG raised in goat (Sigma, T-5993), both diluted 1:200 in PBS-BSA, for 1 h at room temperature. Controls included incubating with secondary antibody only, mouse pre-immune serum and monoclonal anti-thymus-myosin II antibody which cross-reacts against a wide spectrum of cytoplasmic myosin IIs in animal cells (Drenckhahn et al., 1983). DNA was labelled by incubating with DAPI (10 μg ml⁻¹) for 5 min at room temperature. Specimens were examined in a Zeiss Axiosvert using a Zeiss Planachromat or Neofluar 40× or 100×. Micrographs were taken using Kodak Ektachrome 400 ASA film. The labelled sections were also viewed in a confocal laser scanning microscope (Bio-Rad MRC 600) equipped with argon/krypton lasers and dual excitation/emission filter sets for both TRITC and FITC for the double-labelled specimens.

For immunoelectron microscopy 2-day-old cress roots were cut and fixed in 8% formaldehyde and 0.1% glutaraldehyde in PBS for 1 h at room temperature, embedded in a drop of 3.2% sucrose and frozen on metal holders in liquid nitrogen. Ultra-thin sections were cut on an cryo-ultramicrotome (Ultracut UCT, Leica/Reichert-Jung, Germany) fitted with cryo设备 FC-S and transferred onto nickel grids. The sections were blocked against non-specific labelling by incubating in 0.02 M glycine in PBS for 10 min, 2% gelatin in PBS for 5 min and 0.1% BSA (type V, Sigma) +5% FCS in PBS for 10 min. For immunolabelling the sections were incubated at room temperature for 30 min with the primary antibody, affinity-purified anti-ATM1 (diluted 1:50 in PBS/BSA). They were washed with PBS/BSA and then incubated with secondary antibody goat anti-rabbit IgG-10 nm gold conjugate (BioCell, Cardiff, U.K.) diluted 1:50 in PBS/BSA. The sections were washed with PBS and the reaction stabilized with 1% glutaraldehyde in PBS. After washing in distilled water, sections were contrasted with ice-cold 0.4% uranyl acetate in 1.8% methylcellulose. Labelled sections were examined in a Philips EM301 electron microscope at 80 kV.

For the immunolocalisation studies on plasmodesmata (Figure 5c,d,e) a similar protocol was used. Two-day-old maize seeds were cut and fixed in 4% paraformaldehyde in MTBS buffer for 90 min at room temperature. After washing in MTBS and PBS, and dehydration in a graded ethanol series, the tissue was embedded in LR White Resin (Hard grade, Biocell, Cardiff, UK) and left to polymerise at 36°C. Ultra-thin sections were cut on an ultramicrotome and transferred onto formvar coated nickel grids. The sections were blocked with 50 mM glycine, 5% BSA and 5% normal goat serum in PBS for 30 min and washed with wash buffer (WB) (PBS containing 1% BSA and 0.1% gelatin). They were incubated first with anti-ATM1 (diluted 1:50 with WB) at room temperature for 90 min, washed with WB and incubated with the second antibody, goat anti-rabbit IgG-10 nm gold conjugate (diluted 1:50 in WB) for 90 min. The sections were washed with WB and PBS, post-fixed with 3% glutaraldehyde for 15 min, washed extensively with distilled water and contrasted with ice-cold 2% aqueous uranyl acetate and 1% osmium tetroxide (to enhance plasmodesmata visualization). The labelled sections were examined in a Zeiss EM 10 electron microscope at 60 kV.

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