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Recruitment of myosin VIII towards plastid surfaces is root-cap specific and provides the evidence for actomyosin involvement in root osmosensing

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Abstract. The existence of a cell wall–plasma membrane–cytoskeleton (WMC) continuum in plants has long been postulated. However, the individual molecules building such a continuum are still largely unknown. We test here the hypothesis that the integrin-based multiprotein complexes of animal cells have been replaced in plants with more dynamic entities. Using an experimental approach based on protoplast digestion mixtures, and utilising specific antibodies against *Arabidopsis* ATM1 myosin, we reveal possible roles played by plant-specific unconventional myosin VIII in the functioning of WMC continuum. We demonstrate rapid relocation (less than 5 min) of myosin VIII to statolith surfaces in maize root-cap cells, which is accompanied by the reorganisation of actin cytoskeleton. Upon prolonged stimulation, myosin VIII is also recruited to plasmodesmata and pit-fields of plasmolysing root cap statocytes. The osmotic stimulus is the major factor inducing relocation, but the cell wall–cytoskeleton interactions also play an important role. In addition, we demonstrate the tight association of myosin VIII with the surfaces of chloroplasts, and provide an indication for the differences in the mechanisms of plastid movement in roots and leaves of plants. Overall, our data provide evidence for the active involvement of actomyosin complexes, rooted in the WMC continuum, in the cellular volume control and maintenance of spatial relationships between cellular compartments.

Keywords: actin, cell wall–cytoskeleton interactions, myosin VIII (unconventional), organelle movement, osmosensing, plastids, root cap, statolith.

Introduction

All organisms must conform to the physicochemical realms of the surrounding world. At the organismal level, there are many examples of the sensing of and reaction to physical stimuli, such as gravity, touch, wind, regulation of osmotic balance, and, for example, those resulting from mechanical coupling or interactions of the cells inside the multicellular organism (Hamill and Martinac 2001). Depending on the type of cellular organisation, exemplified by animal and plant cells (Peters *et al*. 2000; Wojtaszek 2000), different modes of operation are found. The constituents of plant signalling pathways identified to date include histidine protein kinase-based two-component systems (Hwang *et al*. 2002), mechanosensory ion channels (Ding and Pickard 1993; Shepherd *et al*. 2002), calmodulin and related proteins (Braam and Davis 1990), phospholipids (Zonia and Munnik 2004), and a variety of MAP kinases (e.g. Dixon *et al*. 1999; Mikołajczyk *et al*. 2000). Recent genomic analyses in yeast (Causton *et al*. 2001) and *Arabidopsis* (Moseyko *et al.* 2002; Centis-Aubay *et al*. 2003; Paul *et al*. 2004) identify significant overlaps of gene expression patterns induced by various types of stimuli. This is true for both gravi- and osmosensing coupled with the regulation of cell volume (Brownlee *et al*. 1999) and for

Abbreviations used: CLB, chloroplast liberation buffer; DS, digestion solution; FITC, fluorescein isothiocyanate; NaOAc, 5 mM sodium acetate buffer pH 5.8; PBS, phosphate-buffered saline; RT, room temperature; SB, stabilising buffer; WMC, cell wall–plasma membrane–cytoskeleton.

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gravi-/mechanosensing (Centis-Aubay *et al*. 2003; Paul *et al*. 2004). Despite these overlaps, plant cells are able to discriminate between, for example, hyper- and hypo-osmotic stress (Felix *et al*. 2000; Zonia and Munnik 2004) as well as between osmotic stress and osmotically driven mode of plant infection (Dixon *et al*. 1999).

The existence of plant cell wall–plasma membrane–cytoskeleton continuum, analogous to that of animal cells, has long been postulated (Wyatt and Carpita 1993). The organisation of the cytoskeleton and its interactions with the surrounding extracellular matrix or cell wall are crucial both for the detection of the mechanical stimuli and for the transduction of the resulting signal (Forgacs 1995; Ingber 2003). For example, osmosensing in yeast is tightly coupled with the monitoring of cell wall integrity (Hohmann 2002), and some initial data indicate the existence of similar mechanisms in plants (Marshall and Dumbroff 1999; Nakagawa and Sakurai 2001). In 'walled' plant cells (Baluška *et al*. 2003*a*, *b*), the walls prevent bursting of the protoplast either by counteracting the turgor pressure of the protoplast in normal or hypotonic environment or by providing an anchor for the plasmolysing protoplast under hyperosmotic conditions ('the plasmolytic cycle'; Lang-Pauluzzi and Gunning 2000). Actin microfilaments (Lang-Pauluzzi and Gunning 2000; Komis *et al*. 2002, 2003), Ca^{2+} currents (Brownlee *et al*. 1999; Hayashi and Takagi 2003) and novel ankyrin protein kinases (Chinchilla *et al*. 2003) might also be involved in the control of protoplast's integrity during such cycles.

Although the existence of WMC continuum in plant cells is supported by substantial evidence, the individual molecules building such continuum are still largely unknown (Baluška *et al*. 2003*a*). During our survey, some potential linkers, such as wall-associated kinases (Anderson *et al*. 2001), were highlighted and the role of formins, for example, has been confirmed (Cheung and Wu 2004). The possibility that the integrin-based multiprotein complexes of animal cells have been replaced in plants with much more dynamic entities, has also been indicated (Baluška *et al*. 2003*a*). In this paper, we provide evidence for the role played by plant myosin VIII in the functioning of WMC continuum. Myosins as F-actin-activated ATPases are motor proteins exerting forces along actin filaments and are classified into at least 17 different classes (Bezanilla *et al*. 2003). Among 17 genes coding for myosins in *Arabidopsis* genome, 13 genes belong to class XI, similar to myosin V, and the remaining four are grouped within the plant-specific class VIII of unconventional myosins (Reddy and Day 2001). Myosin-like proteins can be found associated with surfaces of plant organelles, and are probably involved in actomyosin-based cytoplasmic streaming (Yokota 2000) as well as in trafficking and movements of organelles (Holweg and Nick 2004), plastids in particular (Liebe and Menzel 1995; Malec *et al*. 1996). However, in most of the studies heterologous antibodies, directed against total bovine myosin or myosin II from chicken muscle, were used. The use of two anti-myosin XI antibodies revealed two distinct patterns of labelling. An antibody against a myosin XI head domain epitope indicated a punctuate distribution, probably related to mitochondria and 'low-density membrane fraction' (Liu *et al*. 2001). In contrast, the use of an antibody recognising a conserved tail region enabled identification of a subclass of plant myosin XI associated with both mitochondria and plastids (Wang and Pesacreta 2004).

Using the antibody directed against *Arabidopsis* ATM1 myosin, we have demonstrated previously that in untreated cells of the transition zone in maize root apices myosin VIII localised at the plasmodesmata and at the developing cell plates and suggested its role in the actin cable re-establishment (Reichelt *et al*. 1999). As the root cap is the most important osmosensing device of the plant, we decided to check its dynamics with the use of anti-myosin VIII antibody utilising an experimental approach based on hypertonic protoplast digestion mixtures. We demonstrate here that under disturbed mechanical and osmotic conditions myosin VIII undergoes rapid relocation (within less than 5 min) to amyloplast surfaces in root cap cells, and that this relocation is accompanied by the reorganisation of the actin cytoskeleton. Following recent suggestions that the systems for plastid movement in roots and in leaves might differ to some extent (Oikawa *et al*. 2003), we also decided to compare the association of myosin VIII with the surfaces of root amyloplasts and with leaf chloroplasts.

Materials and methods

Plant material

Maize (*Zea mays* L. cv. Careca S230) caryopses were imbibed under running tap water for 16–20 h and germinated in moistened rolls of filter paper for 2 d at 25°C in the dark. For experiments, root apices were excised from straight, 40–70-mm long, primary roots. *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia] plants were grown in pots in an environmental chamber Sanyo MLR-350H (Tokyo, Japan), equipped with fluorescent tubes (Sanyo FL 40SS.W/37), at 10/14 h light/dark photoperiod. Light fluence was 60 μmol m^{-2} s^{-1}.

General layout of experiments

Apical root segments, 4–6 mm, were cut and fully submerged in horizontal position in deionised water. They were kept at room temperature (RT) on a rotary shaker until the start of experiment. Following aspiration of water, root apices (15–20 per treatment) were transferred to 35-mm Petri dishes containing respective solutions (time-point zero), and infiltrated under vacuum for 90 s. They were then incubated in a shaking bath set at 70 rpm and 30°C. Four root segments per treatment were removed at time-points 5, 30, and 90 min, and processed for immunofluorescence microscopy. In all cases, 5 mM NaOAc was used as a control.

Experimental treatments

For the digestion of cell walls, root segments were transferred to 1 mL of 8 mM MES–KOH buffer, pH 5.5 containing 1 mM $CaCl_2$, 0.5% (w/v) polyvinylpyrrolidone, 0.5% (w/v) BSA, 0.8% (w/v) cellulase Onozuka RS (Yakult Honsha; Serva, Heidelberg, Germany),

0.1% (w / v) pectolyase (Sigma, P3026, St Lois, MO), and 0.6 M sorbitol. Following a 1-h incubation, identical volume of the same solution, but without pectolyase and buffered at pH 5.8, was added, and treatment prolonged for the next hour (Kollmeier *et al*. 2001).

The effects evoked by enzymatic or osmotic components of the protoplast preparation mixture were determined using 8 mM MES–KOH buffer, pH 5.8 containing 1 mM $CaCl_2$, 0.5% (w / v) polyvinylpyrrolidone, 0.5% (w / v) BSA. Root apices were incubated in 2 mL of this solution containing either enzymes (Onozuka RS + pectolyase) or sorbitol, respectively, at the same concentrations as above.

To determine the role of root orientation against the gravity vector, excised root apices were oriented either vertically with the root cap pointing up or down, or horizontally. This was achieved by placing the segments along the side walls (root cap up) or at the bottom of Petri dishes and fixing them in these positions with 10-µL droplets of 0.8% agarose type VII, low gelling temperature. Root apices were then oriented in their intended position, immersed in 5 mM NaOAc and left still for 45 min at 25°C to allow for setting of statoliths. For the treatment, buffer was aspirated and root segments were placed in 5 mM NaOAc containing 0.6 M sorbitol (time-point zero) and incubated without shaking at 25°C. An additional experimental variant included changing the orientation of root apices by 180° from 'vertical up' to 'vertical down' at time-point zero to induce movement of statoliths.

For enzymatic digestions, root apices were incubated in 8 mM MES–KOH buffer, pH 5.8 containing 1 mM $CaCl_2$, 0.5% (w / v) polyvinylpyrrolidone, 0.5% (w / v) BSA, 0.6 M sorbitol, and one of the following: 1% (w / v) cellulase (0.3 U mg^{-1}; Sigma C1184), 1% (w / v) hemicellulase (1.58 U mg^{-1}; Sigma H2125), 0.1% (w / v) pectolyase (4.5 U mg^{-1}; Sigma P3026), 30 µg mL^{-1} trypsin (10 900 U mg^{-1}; Sigma T8003) or 30 µg mL^{-1} collagenase (388 U mg^{-1}; Sigma C5138).

Immunocytochemistry

Excised root segments were processed for immunofluorescence microscopy according to a slight modification of the method described by Baluška *et al*. (1992). Briefly, excised root apices were immediately transferred to 4% (w / v) paraformaldehyde and 0.1% (v / v) glutaraldehyde in stabilising buffer (SB; 25 mM PIPES, 2.5 mM $MgSO_4 \times 7H_2O$, 2.5 mM EGTA, pH 6.9) and, after a vacuum infiltration cycle (90 s), fixed for 1 h at RT. Following washing with SB and dehydration in a graded ethanol series diluted with phosphate-buffered saline (PBS), root segments were embedded in low-melting-point Steedman's wax (Baluška *et al*. 1992).

For immunolabelling, 10-µm sections were incubated for 1 h at RT with the following primary antibodies: anti-myosin VIII (*A. thaliana*) polyclonal antibody (Reichelt *et al*. 1999) diluted 1 : 100, and anti-maize pollen actin polyclonal antibody (Baluška *et al*. 2001*a*) diluted 1 : 100 or anti-human actin monoclonal antibody (clone C4; ICN, 691002) diluted 1 : 200. All dilutions were made with PBS containing 0.1% (w / v) BSA. Following rinsing with PBS (10 min), sections were incubated for 1 h at RT with anti-rabbit IgGs, $(Fab')_2$ fragments, conjugated with fluorescein isothiocyanate (FITC; Sigma, F1262), with FITC-conjugated anti-mouse IgGs (Sigma, F9006) or with Alexa 594 anti-rabbit IgGs (Molecular Probes, A11062, Invitrogen, Carlsbad, CA). All secondary antibodies were diluted 1 : 100 with PBS containing 0.1% BSA. A further wash with PBS (10 min) preceded a 10-min staining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; 100 µM in PBS). Following rinsing in PBS (10 min), sections were treated for 10 min with 0.01% (w / v) toluidine blue in PBS to diminish autofluorescence of root tissues. Mounting was done using anti-fading reagent based on *p*-phenylenediamine (Baluška *et al*. 1992). Sections were examined with a Zeiss Axiovert 405M (Zeiss, Göttingen, Germany) inverted microscope. Images were acquired with a Zeiss AxioCam HRc camera operating under AxioVision 3.1 software, and further processed with Adobe PhotoShop. The selection of images was also deconvoluted with a software package from Autoquant (http://www.aqi.com, validated 10 May 2005).

Labelling and immunofluorescence analysis of leaf mesophyll and isolated chloroplasts

One-month-old *Arabidopsis* leaves were used for experiments. After removal of the epidermis, the mesophyll tissue was cut into small squares and infiltrated with 1% (w / v) paraformaldehyde, 1% (v / v) DMSO, 0.2 mM ATP-Na_2 in actin stabilising buffer (ASB; 50 mM PIPES, 10 mM EGTA, 5 mM $MgSO_4$, pH 6.7). The tissue fragments were incubated for 2 h in darkness, at 14°C, with gentle shaking. Fixed samples were washed three times with ASB and incubated with shaking for 1 h at 14°C with an anti-myosin VIII polyclonal antibody (Reichelt *et al*. 1999) diluted 1 : 50 or anti-myosin (smooth and skeletal; Sigma, M7648) diluted 1 : 10. After washing with ASB, the samples were incubated with shaking for 1 h at 14°C with a FITC-conjugated goat anti-rabbit IgGs (Sigma, F0382) diluted 1 : 80, and finally washed with ASB. As a control, samples were labelled either with secondary antibody only or with a rabbit complement (Hoechst-Behring, Mannheim, Germany) diluted 1 : 10 and used as a primary antibody. All dilutions were made with ASB. For preparation of protoplasts, a hypertonic 0.6 M mannitol in 1 mM MES, 10 mM $CaCl_2$, 0.5% (w / v) BSA, 0.5% (w / v) PVP solution containing cell wall digesting enzymes: 1% (w / v) cellulase Onozuka RS, 0.2% (w / v) Macerozyme (Yakult Honsha Co. Ltd, Tokyo, Japan) was used. The cut tissue was infiltrated, the incubation proceeded in darkness for 15 min at 22°C, without shaking, and was followed by fixation and labelling as described above. All samples were scanned immediately after preparation.

Two independent methods of chloroplast isolation from *Arabidopsis* leaves were employed. In the first method, the tissue, following removal of epidermis, was treated with digestion solution (DS) pH 5.8, containing 0.2 mM KH_2PO_4, 1 mM KNO_3, 2 mM $MgSO_4$, 0.9 µM KI, 0.1 µM $CuSO_4$, 0.5 mM MES, 26 mM $CaCl_2$, 2% (w / v) cellulase Onozuka RS, 0.2% (w / v) Macerozyme, and 0.4 M sucrose. The digestion was conducted in the dark, without shaking, for 1.5 h at 28°C. The digested tissue was filtered through a 100-µm nylon mesh and washed in DS solution without enzymes. The osmolality of wash solution was 0.51 mol·kg^{-1} as measured with a 5100C vapour pressure osmometer (Wescor Inc., Logan, UT). The protoplasts were collected by centrifugation at 30 *g* for 10 min, at 4°C. The upper band containing intact protoplasts was removed and washed twice with the wash medium W5 (Menczel *et al*. 1981). In order to liberate intact chloroplasts, the protoplasts were lysed in the CLB (chloroplast liberation) buffer: 25 mM PIPES, 2.5 mM $MgSO_4$, 2.5 mM EGTA, 62.5 mM $CaCl_2$, 2.5 mM KCl, 72.5 mM NaCl, 2.5 mM glucose, pH 6.9, osmolality 0.33 mol·kg^{-1}. Chloroplasts were also isolated from homogenised leaves following the protocol of Aronsson and Jarvis (2002). Intact chloroplasts obtained with a two-step Percoll gradient were suspended in CLB.

For the labelling, isolated chloroplasts were incubated for 1 h at RT with anti-myosin VIII polyclonal antibody diluted 1 : 50 or with anti-myosin (smooth and skeletal; Sigma, M7648) antibodies diluted 1 : 10. Dilutions were made with CLB. After washing with CLB, chloroplasts were incubated for 1 h at RT with FITC-conjugated secondary antibodies (Sigma, F0382), diluted 1 : 80 with CLB. The immunolabelled chloroplasts were collected by centrifugation at 50 *g* for 10 min at 4°C, and mounted on slides. Myosin was visualised in the Confocal Laser Scanning Microscope BioRad MRC 1024 (BioRad, Hercules, CA). Fluorescence was excited by blue light from argon laser at 488 nm, and observed in two channels. FITC fluorescence was shown in the green channel, with the filter 540 DF30, and the autofluorescence of chloroplasts in the red channel, with filter 585LP.

Results

Localisation of myosin VIII in control root apices

To demonstrate typical localisation of myosin VIII in root cap cells, maize root apices from vertically grown plants were cut and, following immediate fixation and embedding, used for immunofluorescence labelling. In the roots of 3-d-old seedlings, the relatively weak myosin VIII signal is diffusely distributed with fairly large proportion of the protein located at the nuclear surfaces. Quiescent centre cells are extremely weakly labelled while stronger signal is scored in the peripheral cells of the root cap (Fig. 1*A*). This pattern of labelling is significantly different from that seen in the cells of the maize root transition zone where, in agreement with our previous data (Reichelt *et al*. 1999), the antibody labels plasmodesmata of the cross-walls and the developing cell plates (see e.g. Fig. 2*A*). These data suggest that, in maize, the mechanical integrity of the root caps is maintained separately from that of other portions of root apices.

During preparation of protoplasts the roots were usually kept on an orbital shaker. Thus, the major concern here was to exclude from further considerations possible relocations of myosin VIII invoked only by a permanent mechanical disturbance of statoliths and / or amyloplasts within root cells. To monitor changes in myosin VIII distribution evoked by shaking, control root apices, submerged in 5 mM sodium acetate buffer, pH 5.8 (NaOAc), were treated in the same way as excised root apices subjected to experimental protocol. When compared with normal myosin VIII distribution (Fig. 1*A*), two significant changes in the labelling pattern in control root apices were detected. First, the label became more focused (Fig. 1*C*), and the strength of labelling increased with duration of incubation. Second, the punctuated myosin VIII labelling appeared in few cell types, revealing a very characteristic, ring-like pattern around nuclei, especially in the peripheral root cap cells and in some cell files of the root meristem (Fig. 1*B*).

Myosin VIII relocates to statolith surfaces upon cell wall degradation from root cap statocytes

Our initial experiments aimed at generation of protoplasts from different growth zones of maize roots revealed that normal localisation of myosin VIII changed under conditions

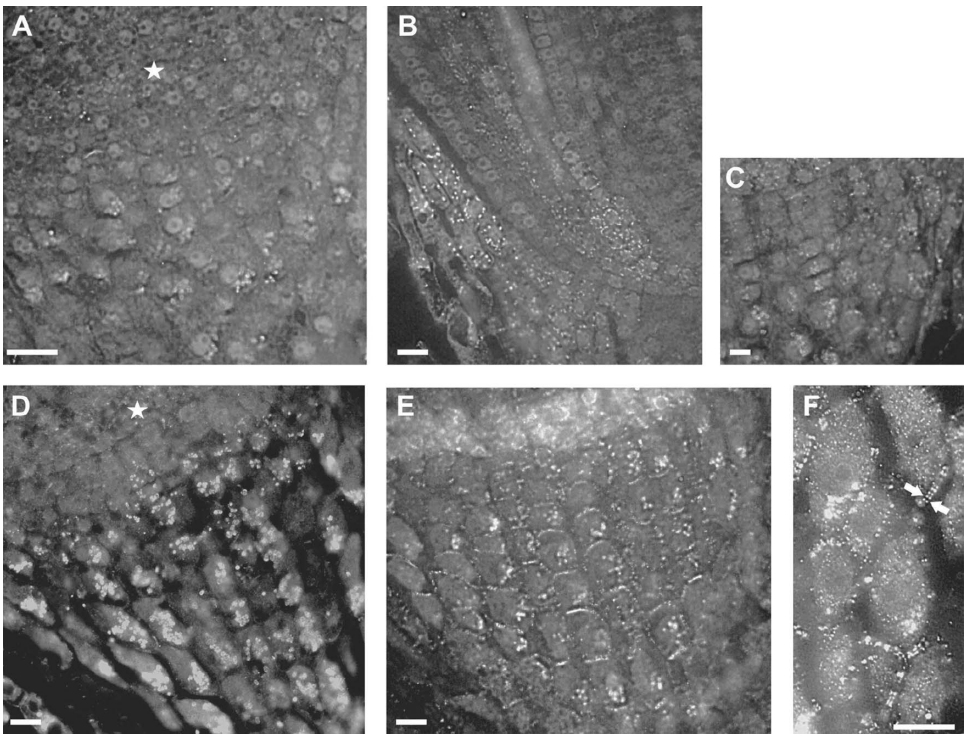

Fig. 1. Immunofluorescence localisation of myosin VIII in root caps of maize roots subjected to protoplast preparation. Myosin VIII is poorly detected in the root caps of vertically-grown plants (*A*). In control roots incubated in NaOAc buffer only, the labelling becomes slightly more pronounced and localised after 90 min of treatment (*B*, *C*), especially in peripheral cells of the root cap (*B*). During protoplast preparation (*D–F*), myosin VIII rapidly relocalises to amyloplasts in root cap cells (*D*, 5 min after initiation of treatment). Prolonged protoplasting up to 90 min does not change this pattern of labelling, but induces recruitment of myosin VIII to plasmodesmata connecting root cap cells (*E*, *F*). ⋆(*A*, *C*) indicate the position of quiescent centre; arrows in (*F*) points to both entrances to the plasmodesmata. Scale bars indicate 20 µm.

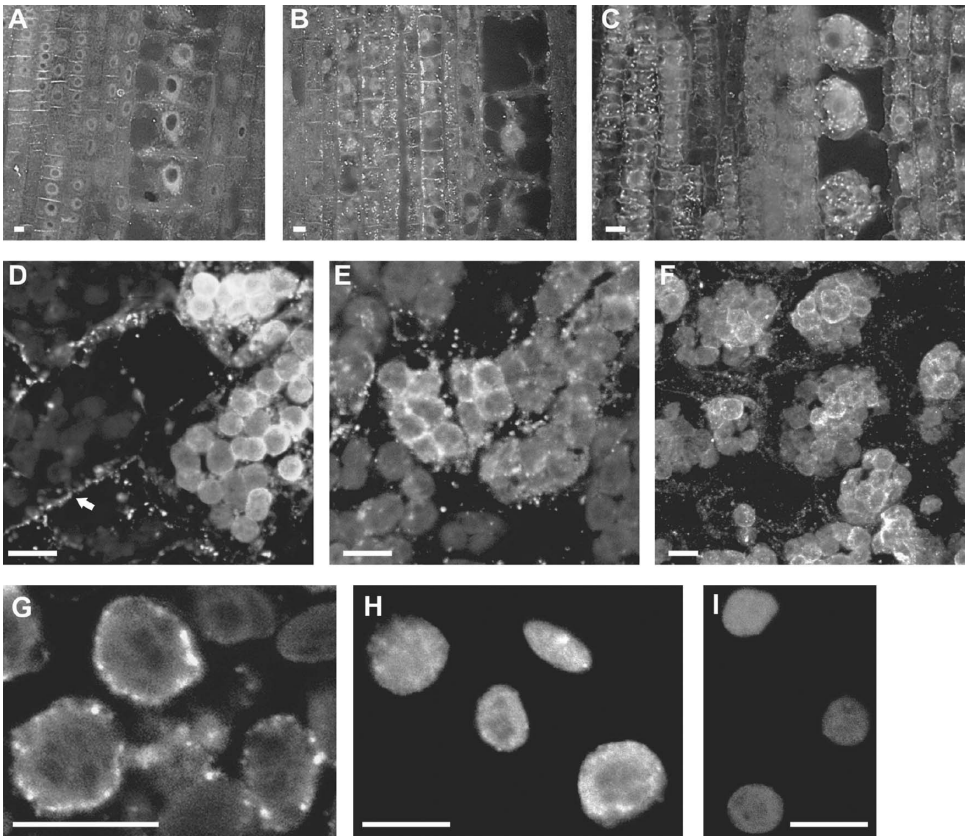

Fig. 2. Comparison of the patterns of association of myosin VIII with plastid surfaces in maize roots (*A–C*) and *Arabidopsis* leaves (*D–F*) *in situ* and *in vitro*. In transition and elongation zones of control root apices (*A*), myosin localises mainly at the cross-walls and newly formed cell plates. During protoplast preparation, myosin VIII gradually relocalises towards plastid surfaces (*B*, at 5 min; *C*, at 90 min), similarly like in the root cap cells. In *Arabidopsis*, myosin-like proteins are detected on the chloroplast surfaces of both control (*D*, *E*) or protoplasted (*F*) leaves with antibodies against bovine myosin (*D*) and with anti-myosin VIII antibodies (*E*, *F*). Note the appearance of labelling at the plasmodesmata along leaf cell walls (*D*, arrow). Myosin seems to be more permanently associated with chloroplasts as the same patterns of labelling with anti-bovine myosin (*G*) and anti-myosin VIII antibodies (*H*) can be found on organelles isolated from leaves. No labelling could be seen in control treatments exemplified here with chloroplasts labelled with rabbit complement instead of primary antibody (*I*). Scale bars indicate 20 µm (*A–C*) and 10 µm (*D–I*).

producing protoplasts. This response was particularly strong in the root-cap cells. To examine this phenomenon more thoroughly, a time-course analysis was performed, during which root apices were sampled from the protoplast preparation mixture at different time-points and used for immunofluorescence localisation of myosin VIII. The earliest time-point at which generation of repeatable data was technically feasible was usually at 5 min after placing the roots in the protoplast isolation cocktail. At that sampling point, the relocation of myosin VIII was clearly visible both in the root caps (Fig. 1*D*) as well as in other parts of the roots (compare Fig. 2*A*, *B*), indicating a very rapid response. The initially dispersed myosin VIII signal (Fig. 1*A*) was converted into a highly localised signal with the surfaces of subcellular compartments, and of the statolith surfaces in columella cells emerging as sites of strong myosin VIII signal (Fig. 1*D*). Prolonged exposure to the protoplast preparation medium did not dramatically change this pattern (Fig. 1*E*, *F*). However, longer incubation induced plasmolysis of the root-cap protoplasts resulting in recruitment of myosin VIII to plasmodesmata and pit-fields. At that time (90 min), the pattern of labelling in the root cap cells became similar to that noted in normal cells of the transition and elongation zones of maize roots (Reichelt *et al*. 1999). The labelling was extremely prominent at the plasmodesmata of transverse walls (Fig. 1*E*) and also, but not always, along other walls of root cap cells (Fig. 1*F*). Interestingly, the punctuated appearance of myosin VIII labelling in plasmolysed root

cap cells clearly demonstrates that the protein is not evenly distributed along the plasmodesmal channel, but its concentration is much higher at both entrances to the plasmodesmata.

Relocation of myosin VIII in maize roots is different from that in Arabidopsis *leaves*

Preliminary analyses of maize root caps demonstrated that under normal conditions anti-myosin VIII antibody did not label amyloplast surfaces (Volkmann and Baluška 2000), which is in agreement with our present data (Fig. 1*A*). Similarly, as in root caps, plastids / amyloplasts are not normally labelled in cells of other developmental zones of maize roots (Fig. 2*A*), but myosin VIII becomes rapidly relocalised towards their surfaces upon production of protoplasts (Fig. 2*B*, *C*). As recent data on CHUP1 protein (Oikawa *et al*. 2003) suggest that the systems for plastid movement in roots and in leaves might differ to some extent, we compared the observable patterns of myosin VIII relocation in maize roots with those seen in *Arabidopsis* leaf spongy mesophyll cells. Two types of antibodies were applied: plant myosin VIII antibodies and anti-bovine myosin antibodies, previously demonstrated to label chloroplasts isolated from *Lemna trisulca* (Malec *et al*. 1996). In normal fixed cells, myosin VIII was found on the surface of chloroplasts and at the cell periphery (Fig. 2*E*). Twin points visible on several images suggest that at least part of the label matches with plasmodesmata (data not shown). No specific localisation of myosin VIII around the nucleus was detected. In the samples treated with the anti-bovine myosin antibodies, fluorescent spots were considerably more numerous (Fig. 2*D*). After cell wall degradation, the localisation of myosin VIII was similar to that observed in normal cells with more intense labelling and greater number of spots on the cell walls (Fig. 2*F*).

To get a better insight into the nature of myosin VIII interactions with plastids, we decided to isolate chloroplasts from *Arabidopsis* leaves and to check whether or not myosin VIII antibodies would still recognise the corresponding epitopes. Importantly, the binding of myosin VIII to the surface of chloroplasts was strong enough to survive isolation procedures. Two independent methods of chloroplast isolation were employed, gentle osmotic shock of isolated protoplasts and / or fractionation of homogenised leaves. The results were similar for both types of antibodies, irrespective of the mode of chloroplast isolation. In all cases, chloroplast surfaces were decorated with dispersed, punctuated label (Fig. 2*G*, *H*) that sometimes appeared as intense bright spots suggestive of multiprotein complexes. Similar to the images from the cell periphery, the fluorescence of chloroplast envelopes was noticeably more intense when antibodies against animal myosin were used. All controls gave reproducible data showing no labelling of chloroplasts (Fig. 2*I*). Overall, these results suggest that other species of myosin, maybe of myosin XI (Wang and Pesacreta 2004), might be present at the cell periphery and / or at the chloroplast outer membrane.

Osmotic stimulus is the major cause of rapid myosin VIII relocation in root cap cells

In a search for identification of possible mechanisms inducing rapid relocation of myosin VIII in maize root caps, we first took a closer look at the composition of the protoplast isolation cocktail. Among its components, sorbitol and cell wall-degrading enzymes (cellulase Onozuka RS and pectolyase) seemed to be potential candidates for inducers of the reorganisation of cellular architecture. The former via creating hyperosmotic conditions, the latter as factors disturbing the mechanical stability of the walls and, consequently, of the whole cells. We compared the changes in patterns of myosin VIII labelling during the process of isolating protoplasts (Figs 1, 3*A*) with those observed in root caps subjected to treatments with protoplast preparation mixtures based on identical components, but lacking either sorbitol or cell wall-degrading enzymes.

Cell wall-degrading enzymes alone were not able to induce relocation of myosin VIII. Labelling was poor and remained dispersed throughout the period of incubation (Fig. 3*B*, *C*). Slightly more focused labelling was observed in cells of the root apex, similar to that in control roots subjected to continuous mechanical stimuli (compare Figs 3*C*, 1*B*). In contrast, when the osmotic agent was used alone, both the speed of changes and the patterns of myosin VIII labelling were very similar to those identified in root apices subjected to protoplast preparation. After 5 min, the altered labelling of statoliths / amyloplasts in columella and other root cap cells was already clearly visible (Fig. 3*D*). This was vastly different from the pattern of labelling seen in sloughing root-cap cells where myosin VIII antibodies strongly decorated nuclei and, to some extent, the cytoplasm (Fig. 3*E*). In root apices sampled at 90 min, plant responses to protoplast preparation or to osmoticum alone were almost indistinguishable (compare Fig. 3*A*, *F*). We thus conclude that the relocation of myosin VIII to statolith surfaces is part of the root cap cell response to hyperosmotic conditions, probably evoked by changing cellular volumes.

Relocations of myosin VIII are accompanied by the reorganisation of actin cytoskeleton

Regarding these rapid relocations of myosin VIII, at least two mechanisms can be envisioned. First, myosin VIII could rapidly move along existing actin filaments towards statolith surfaces. Second, myosin VIII movement might be accompanied by the active reorganisation of actin cytoskeleton involved in the positioning of statoliths inside root cap cells. Therefore, double immunolabelling experiments were performed with anti-actin and

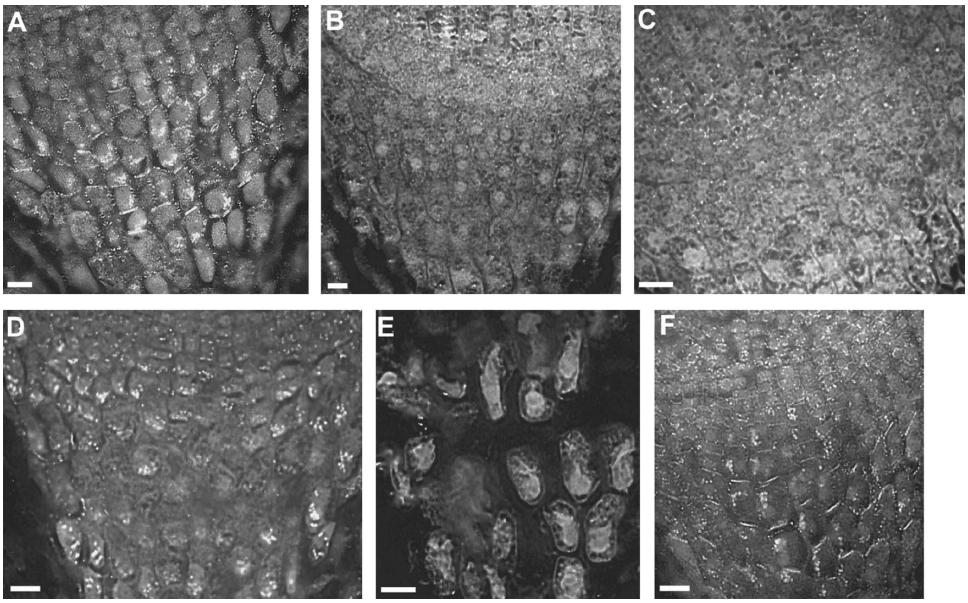

Fig. 3. The role of various components of the protoplast isolation mixture in the induction of rapid relocations of myosin VIII. (*A*) Immunofluorescence localisation of myosin VIII in root caps treated with protoplast isolation mixture for 90 min. In the maize root cap cells subjected to treatment with enzymatic components (without osmoticum), myosin VIII labels poorly (*B*, at 5 min) or the label remains dispersed (*C*, at 90 min). When root apices are treated with buffered 0.6 M sorbitol (without wall-degrading enzymes), myosin VIII is either localised in columella cells (*D*), or strongly dispersed in sloughing root cap cells (*E*) as early as 5 min after treatment. Following 90 min of osmotic treatment, the labelling of plasmodesmata is also clearly visible (*F*). Scale bars indicate 20 μm.

anti-myosin VIII antibodies (Fig. 4). Our earliest possible images, taken about 2 min after the initiation of cell wall degradation, show strongly focused location of myosin VIII around statoliths. Interestingly, the strength of labelling seems to be dependent on the position of individual cells inside the root cap with stronger labelling at root cap peripheries and weaker labelling in the deep root cap interior (Fig. 4*C*). This probably reflects the progress of osmotic stimuli with external root cap cells exposed to hyperosmotic conditions for a relatively longer period compared with the cells inside the root cap. This short exposure time is also sufficient to induce reorganisation of actin from the normally dispersed pattern (Fig. 4*A*) to a more focused pattern (Fig. 4*B*). When the labelling patterns of myosin VIII and actin are overlaid, co-localisation of both proteins becomes apparent (Fig. 4*D*).

Myosin VIII relocations are accomplished irrespective of the gravity vector

The actin cytoskeleton is implicated in the gravisensing phenomenon, linking the positioning of statoliths with specialised mechanosensitive zones within statocytes (Braun 2002). Therefore, we determined whether or not the relative orientation of the statocytes against the gravity vector is important for the observed specific relocation of myosin VIII induced by osmotic stimulation. Excised root apices were oriented and stabilised in different positions for 45 min under a layer of the NaOAc buffer to allow statoliths to settle. The experiment was initiated with the aspiration of the buffer and addition of 0.6 M sorbitol in NaOAc. A further experimental variant involved repositioning of root apices, inverted by 180° at the beginning of osmotic stimulation, to induce movements of statoliths within root cap cells. It should be stressed that, contrary to previous experiments, these root apices were kept still, without any additional movement induced e.g. by the rotary shaker.

Analysis of images taken from control root apices collected five minutes after the initiation of the experimental treatment indicated that 45 min of stabilisation was sufficient for the statoliths to settle. No involvement of myosin VIII in statolith movement was apparent as the labelling with myosin VIII antibodies in all samples and experimental variants was diffused and not directly related to statolith surfaces (Fig. 5*A*, *B*, *E*, *F*, *I*, *J*). Prolonged incubation in buffer did not change the pattern of labelling (Fig. 5*K*, *L*, *N*, *O*). These data correlate well with those presented earlier (Fig. 1) and suggest that there is no relocation of myosin VIII in the absence of external mechanical stimulation induced e.g. by shaking. Identical conclusion could be made for statoliths / amyloplasts moving throughout the cells of inverted root apices, i.e. rotated by 180° (Fig. 5*I*–*L*).

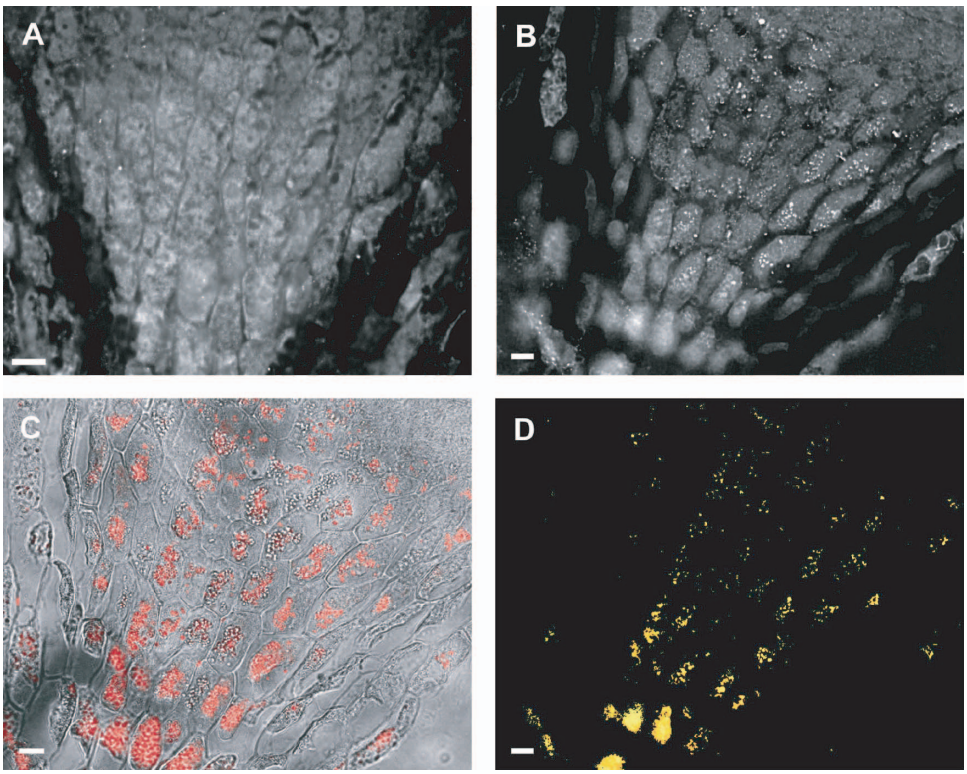

Fig. 4. Double-labelling immunofluorescence localisation of actin and myosin VIII in the root caps of maize roots treated with protoplast isolation mixture. In control root apices, the labelling of actin in the root cap cells appears diffused (*A*). Following the application of the protoplast isolation mixture, microfilaments reorganise and become slightly focused (*B*). At the same time, myosin VIII relocates to amyloplast surfaces as demonstrated by the overlay of myosin VIII labelling onto DIC image (*C*). Note the less pronounced labelling in central root cap cells reflecting the progress of penetration of protoplast isolation mixture. The existence of actomyosin complexes in maize root cap cells is shown in the co-localisation image (*D*). All images taken from samples collected approximately 2 min after initiation of protoplast preparation. Immunofluorescence double-labelling performed with polyclonal anti-myosin VIII and monoclonal anti-human actin antibodies. Scale bars indicate 20 µm.

In root apices subjected to osmotic stimulation, in every orientation of the roots against the gravity vector, myosin VIII was quickly relocated to statolith surfaces, and the intensity of myosin VIII labelling was slightly increased with prolonged osmotic treatment (compare Fig. 5*C*, *D*; Fig. 5*G*, *H*). Moreover, identical pattern of labelling was observed in inverted root apices (Fig. 5*M*). This might seem surprising as in this experimental variant not only were statoliths repositioning, but also orientation and location of nuclei was taking place in the opposite direction to statolith movement. Therefore, we conclude that the relocation of myosin VIII is not induced by the movement of statoliths throughout the cell, but probably by the concerted action of actomyosin complexes responding to cellular shrinkage induced by osmotic treatment.

The cell wall–cytoskeleton connections and the relocation of myosin VIII

In animal cells, cell shape is regulated and maintained by the interactions of the cytoskeleton with extracellular matrix, occurring mostly at focal adhesions. The shape of plant cells results mainly from the interplay between the internal turgor and the mechanical stability of cell walls (Peters *et al*. 2000). In hyperosmotic conditions, the protoplast shrinks (Oparka 1994), but the connections with surrounding walls are maintained (Lang *et al*. 2004). Myosin VIII was proposed previously to be involved in gating of plasmodesmata and / or restoration of functional actin filaments at sites of intercellular communication (Reichelt *et al*. 1999). Thus, we hypothesise that the quality and quantity of cytoskeleton–cell wall connections might affect the extent of plasmolysis of root-cap cells and thus, indirectly, influence the speed and / or intensity of the myosin VIII relocation in those cells.

To address this possibility, two experiments were conducted. First, root apices were pre-incubated with and then subjected to a process of protoplast isolation in the presence of peptides containing arginyl-glycyl-aspartic (RGD) motif, known to interfere with the formation and maintenance of WMC continuum (P Wojtaszek, F Baluška,

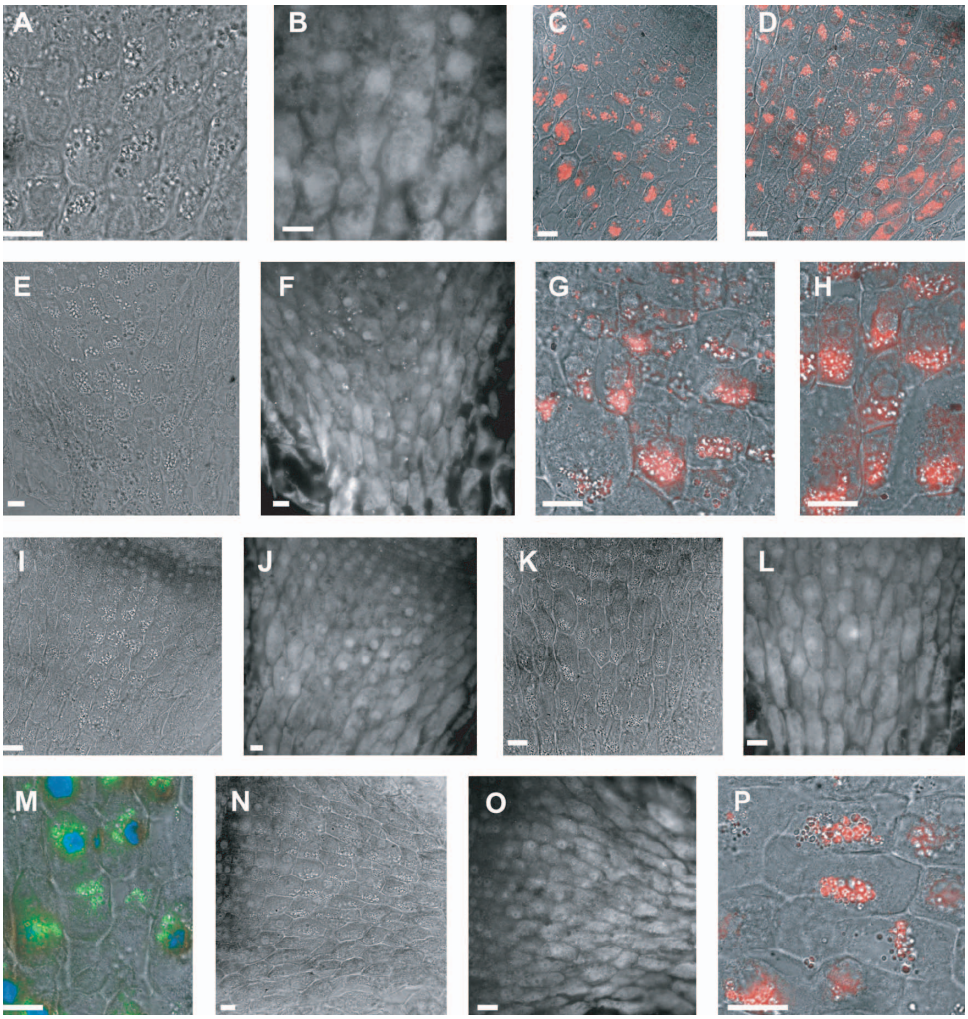

Fig. 5. Osmotically-induced relocation of myosin VIII in roots oriented differently with respect to the gravity vector. Root apices were oriented with their root caps facing up (*A–D*), facing down (*E–H*), reoriented at the moment of applying osmoticum from vertical up position to vertical down to induce movement of statoliths (*I–M*) or horizontally (*N–P*). Control roots were oriented in the same way, but the buffer was used instead of osmoticum. In all orientations, control treatments did not induce relocation of myosin VIII when samples collected at 5 min (*A*, *B*, *E*, *F*, *I*, *J*) or 90 min (*K*, *L*, *N*, *O*) are analysed. However, in the presence of osmoticum relocation of myosin VIII to statoliths' surfaces is very rapid and independent of root orientation. This can be seen as early as 5 min after application of osmoticum (*C*, *G*, *M*, *P*) and the reaction lasts longer, as exemplified by merged images taken at 30-min time point (*D*, *H*). Note that the distribution of moving statoliths also depends on the spatial obstacles inside the cells, e.g. centrally positioned nuclei (*M*; blue, DAPI-stained nuclei; green, myosin VIII labelling). Scale bars indicate 20 µm.

D Volkmann unpubl. data). A second approach involved a comparative analysis of the relocation patterns of myosin VIII induced by full protoplast preparation mixture containing cellulase Onozuka RS and pectolyase, with the root-cap cell responses to enzymatic digestions carried out with individual hydrolytic enzymes in the presence of the osmoticum. It was assumed that the use of more specific carbohydrate- and protein-hydrolysing enzymes should provide an indication for a role of respective cell wall components more directly involved in the formation of WMC continuum. The general pattern of the myosin VIII relocation to amyloplast surfaces and to plasmodesmata at cross-walls was similar for all enzymic treatments (Fig. 6), again indicating that the osmotic stimulus plays a decisive role here. However, in root samples treated with pectolyase or collagenase (see Fig. 6*E*, *G*, respectively), the labelling was slightly different with a punctuated appearance of myosin VIII label at the plasmodesmata located along longitudinal walls. This might suggest that cell wall–cytoskeleton interactions involving pectins and / or proteins,

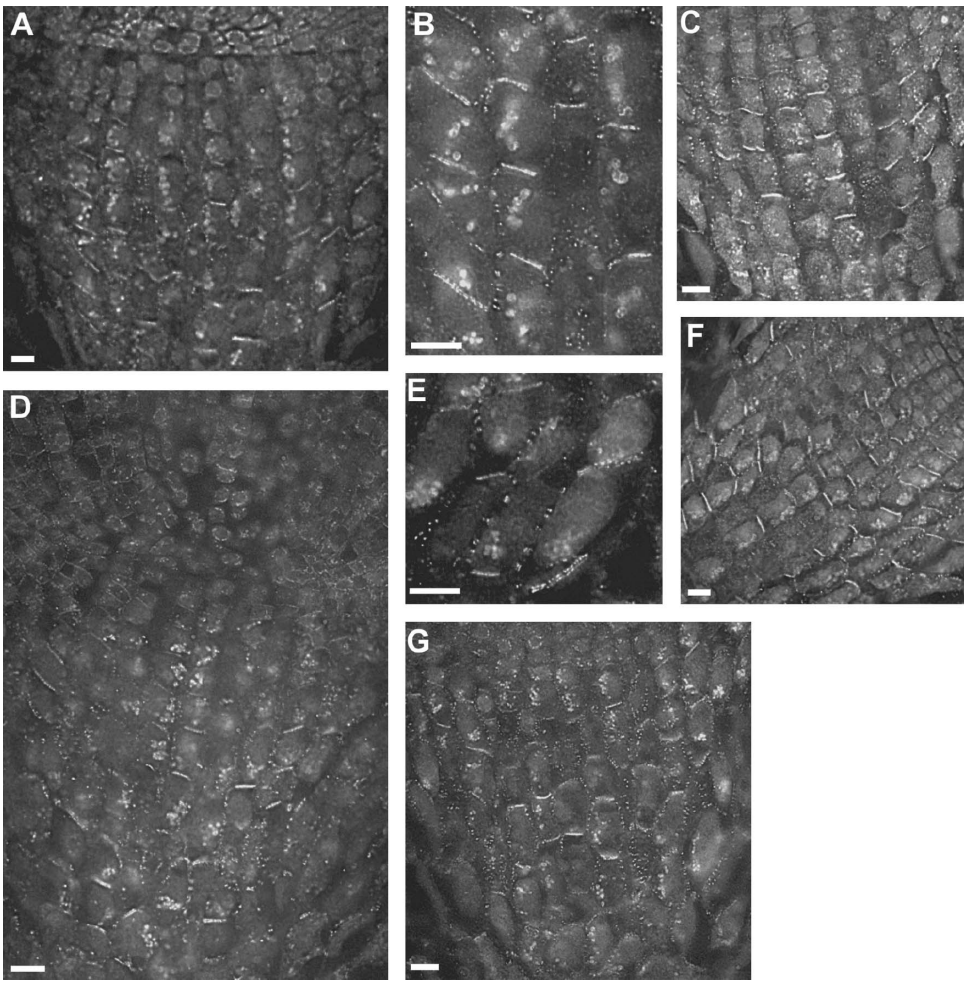

Fig. 6. Various components of extracellular matrix and / or cell wall-plasma membrane-cytoskeleton continuum affects the mechanical stability of the cells resulting in rapid relocation of myosin VIII. Root caps were treated with modified protoplast isolation mixture containing defined enzymes other than those used normally for preparing protoplasts. (*A*, *B*) Cellulase; (C) hemicellulase; (*D*, *E*) pectolyase; (*F*) trypsin; (*G*) collagenase. All images taken from the root caps treated for 90 min. Scale bars indicate 20 μm.

either directly or through further interactions with pectins, for example, are important for the signalling-mediated maintenance of cellular shapes and / or signalling.

Discussion

The root cap is the most important part of a plant to act as an osmosensing device. During the penetration of the soil, it first comes into contact with the surrounding environment and must respond to the rapidly changing osmotic conditions. In agreement with this, our data reveal that osmotic stress induces rapid subcellular redistributions of plant-specific myosin VIII in root cells, especially in gravisensing root-cap statocytes, but not in leaf cells. Relocation of myosin VIII towards various subcellular compartments in response to osmotic stimuli is time-dependent. Myosin VIII in root cap statocytes is normally dispersed, but becomes dramatically and rapidly (within 5 min), focused at the statolith surfaces. Upon progressing plasmolysis of the root cap protoplasts, myosin VIII becomes also localised initially at plasmodesmata of cross-walls and then longitudinal walls. Relocation of myosin VIII is accompanied by the remodelling of actin filaments and, probably, rearrangements of other protein complexes.

Myosin VIII as a putative mechanosensitive cytoskeleton–cell wall linker of plant cells?

Plant cells lack integrins and obviously use other plant-specific WMC linkers (Baluška *et al*. 2003*a*). Among these linker molecules, unconventional myosin VIII emerges as a highly probable candidate. Previous data localised myosin VIII to cytokinetic cell plates and plasmodesmata and pit-fields of the cells in maize root transition zone

(Baluška *et al*. 2001*b*). These subcellular domains are enriched with both callose and pectins, known to have cell-to-cell adhesion properties (Lord and Mollet 2002), and appear to act as platforms for endocytosis and recycling (Baluška *et al*. 2004). Plasmodesmata might act as mechanosensors (Oparka and Prior 1992) and our recent data reveal that gravistimulation controls gating of plasmodesmata in root-cap statocytes optimised for highly effective sensing of the gravity vector (F Baluška, U Tirlapur, D Volkmann unpubl. data). Here, we document that, in mechanically stressed root cells exposed to prolonged osmotic stimulus as well as to enzymes disturbing the mechanical stability of cell walls, peripheral myosin VIII localises especially at plasmodesmata and pit-fields. As myosin VIII is localised to callosic cell periphery domains, we advance a testable proposal that myosin VIII, directly or indirectly, interacts with or participates in the stabilisation of the plasma membrane-based callose synthesis machinery. If this scenario is correct, then peripheral myosin VIII might connect plant adhesion domains based on active callose deposition with the actin cytoskeleton that typically associates with such domains (Baluška *et al*. 2003*a*; Sivaguru *et al*. 2003). Consistent with a link between callose deposition and intact actin cytoskeleton, depolymerisation of actin filaments inhibits callose deposition in pathogen-infected plant cells (Kobayashi *et al*. 1997; Kobayashi and Hakuno 2003).

Myosin VIII and organ-specific organelle movement

A contractile actomyosin system is the basis of cytoplasmic streaming and of organelle motility in plant cells. Of the two components, the involvement of actin has been more thoroughly characterised. Simultaneous visualisation of peroxisomes and microfilaments / microtubules revealed that, contrary to animal cells, plant peroxisomes move using actin filaments (Jedd and Chua 2002; Mathur *et al*. 2002). Active movement of mitochondria was also shown to be actin-based, with microtubules serving as tracks for docking mitochondria in the cortical layer of cytoplasm (Van Gestel *et al*. 2002). Recently, ordered repositioning of mitochondria, endoplasmic reticulum and chloroplasts occurring before cell division was shown to depend on intact actin cytoskeleton in BY2 tobacco protoplast culture (Sheahan *et al*. 2004). Actin filament inhibitors caused disruption of organelle distribution and affected their content in daughter cells. Association of myosin with the surface of mitochondria was reported in wheat (Zhao *et al*. 1999). However, the myosin involvement in the active movement of these organelles was so far indicated only by inhibitor studies (Van Gestel *et al*. 2002) and requires further investigations. Similarly, the role of myosin in the movement of peroxisomes needs clarification as the application of the myosin ATPase inhibitor, 2,3-butanedione monoxime in *Arabidopsis* gave contradictory results (Jedd and Chua 2002; Mathur *et al*. 2002).

The actomyosin system is also active in light-induced chloroplast movements as demonstrated by analyses of glycerinated cell models, effects of myosin inhibitors (Malec *et al*. 1996; Kandasamy and Meagher 1999), and images of actin network, in particular the tight association of F-actin with chloroplasts in *Arabidopsis* (Kandasamy and Meagher 1999). In bryophytes, a microtubule-based system was shown to co-operate with the microfilament-based one in mediating the light-induced movements (Sato *et al*. 2001). In contrast, in higher plants there is no indication that microtubules contribute to chloroplast positioning as a part of force-generating mechanism. The first evidence that a putative motor protein is associated with the surface of chloroplasts was provided by Liebe and Menzel (1995) for *Vallisneria* and Malec *et al*. (1996) for *Lemna trisulca*. However, in both cases heterologous antibodies against animal myosins were used. Here, we show for the first time the association of plant-specific unconventional myosin VIII with the surface of *Arabidopsis* chloroplasts isolated with two independent methods, and of those residing in the leaf mesophyll tissue. In contrast to the root cells, the localisation of myosin in the cells of leaf mesophyll appears to be stable and does not undergo any rapid changes under protoplast-producing conditions that comprise osmotic stress. This evidence clearly supports the idea that systems for plastid movement differ between roots and leaves (Oikawa *et al*. 2003).

Cell wall–cytoskeleton interactions in mechanosensing

Interactions of plant protoplasts with surrounding walls are crucial for their survival and often regulate their fate and development (Brownlee 2002). Although there is reasonable evidence for the functional existence of WMC continuum in plants, the actual linker molecules remain in most cases elusive (Baluška *et al*. 2003*a*). In this paper, we propose the application of protoplast preparation to studies of cellular architecture, both at the single cell and supracellular level, which is based on the following rationale. In animals, the physical state of the extracellular matrix, as well as its chemical composition, regulates the structure and cytoskeletal organisation of cell-matrix adhesions (Katz *et al*. 2000). Moreover, the evidence indicates that mechanical stresses can be transmitted between animal cell types and this results in the respective extracellular matrix remodelling (Swartz *et al*. 2001). In plants, the cell walls, in the form of the apoplast, function as a unique linker between cellular and organismal levels of biological organisation (Wojtaszek 2000, 2001). The stability of 'walled' cells results from an interplay between internal turgor pressure of the protoplast and mechanical stability of the walls (Peters *et al*. 2000). Thus, protoplast isolation, with plant cell stability-disturbing components, might be a technique highly useful for the identification of elusive WMC linker molecules. The advantages include (1) the localised, extracellular site of

action of hydrolytic enzymes used alone or in mixtures; (2) the possibility of selective digestion of macromolecules and / or intermolecular covalent cross-links in the walls; and (3) combination with other agents, like osmoticum, with inhibitors or with intervening molecules, like RGD peptides. We believe that this technique offers a unique mixture of variables affecting either turgor mechanics or mechanical stability / composition of the walls or both (Fig. 7; see also below). At the present stage of knowledge, a more indirect way of reasoning, resulting from the lack of well-developed models of WMC continuum, might be a disadvantage but also a challenge for future studies, e.g. utilising some well-known cell-wall mutants.

In maize, the root cap is to some extent mechanically disconnected from the remaining root body. As gene expression patterns induced by various mechanical stimuli significantly overlap (Brownlee *et al*. 1999; Moseyko *et al.* 2002; Centis-Aubay *et al*. 2003; Paul *et al*. 2004), we believe that our system provides an excellent tool to discriminate cellular responses specific for gravi- and osmosensing. There are two models for the detection of gravitational stimulus in root statocytes. According to the starch–statolith hypothesis, this process is mediated by the sedimentation of specialised amyloplasts (statoliths) inside the statocytes (Sack 1991). Statolith–cytoskeleton interactions, especially with actin, are crucial here, although their extent is still the matter of debate (Baluška and Hasenstein 1997; Yoder *et al*. 2001). In the hydrostatic model, the whole protoplasts act in the similar way to statoliths, i.e. through the generation of differential mechanical stresses in plasma membrane domains at opposite ends of root statocytes (Staves 1997). In our view, the major difference between both models is that the latter one points to the importance of the surrounding cell walls, and WMC continuum in particular (Wayne *et al*. 1992), in detection and transduction of gravitational stimuli, a feature not immediately obvious in models based on the starch–statolith hypothesis. To sense the stimulus, the protoplast needs to be anchored to the surrounding walls via flexible WMC complexes. The differential mechanical signals are then transduced (Soga *et al*. 2005), most probably through the changes in tensegral / percolation-based organisation of cytoskeleton (Forgacs 1995; Ingber 2003). In our system, protoplast preparation, and osmoticum in particular, creates a situation where the stability of WMC continuum is disturbed, thus enabling a direct study of WMC involvement in mechanosensing.

Actomyosin involvement in osmosensing and cell volume regulation: a model

We suggest that our data indicate the existence of a cross-talk between regulatory mechanisms involved in cell volume control, organelle movement and sensing of mechanical stimuli. We propose the following model stressing the importance of three key players: actin filaments, myosin VIII motors, and putative counterparts at the WMC continuum (Fig. 7). In non-stimulated root-cap cells, the dense actin meshwork permeates the whole cell, stabilising its intracellular organisation, among which location of organelles is of prime importance. At least some actin filaments are rooted in WMC continuum to enable oriented reorganisation of actin and / or direct transduction of mechanical signals (Forgacs 1995; Ingber 2003). Such arrangement provides the protoplast with the machinery for fine tuning of cellular shape in a given mechanical environment (Peters *et al*. 2000; Wojtaszek 2000). Maintenance of the large organelle (nucleus) location within the protoplast requires the involvement of myosin VIII, but smaller organelles, like statoliths / amyloplasts, are positioned more passively in the dense actin meshwork.

In our system, three constituent variables are indicative of the role played by individual molecules, and these are: osmoticum, hydrolytic enzymes, and shaking of the excised root apices. In the absence of the latter, positioning or intracellular movement of organelles, although actin-dependent, seem not to engage myosin VIII activity. When the root apices are shaken, the differential oscillations of the organelles are induced and the degree of oscillation reflects the differences of the buoyancies between organelles and the cytoplasm. This phenomenon evokes active, although somehow limited, involvement of myosin VIII (compare Figs 1, 5). It seems to us that the crucial difference here is the appearance of additional mechanical stresses at the WMC continuum induced by shaking of the roots, and direct transduction / transformation of such signals into activation of myosin VIII.

Upon exposure to the protoplast isolation mixture, two important processes are taking place simultaneously, and these are cellular shrinkage induced by osmoticum and weakening of the walls caused by hydrolytic enzymes. The effects are twofold. The cellular volume is gradually reduced and the WMC continuum is placed under an extreme tensile stress. This induces actin rearrangement aimed at maintenance of functionality of subcellular compartments and stabilisation of cellular peripheries (Komis *et al*. 2002, 2003). The latter requires active anchoring of microfilaments at the plasma membrane–cell wall interface, and this is probably achieved through the action of myosin VIII (Fig. 7). Indirect but strong support for this proposal came from recent studies on animal cells, where the unconventional myosin X was found to provide a motor-based link between the cytoskeleton and integrins at the cell peripheries (Zhang *et al*. 2004). Reduction of cellular volume results in changes of the intracellular architecture (Oparka 1994). In root statocytes, one of the consequences would be the decreasing distances between the elements of plant

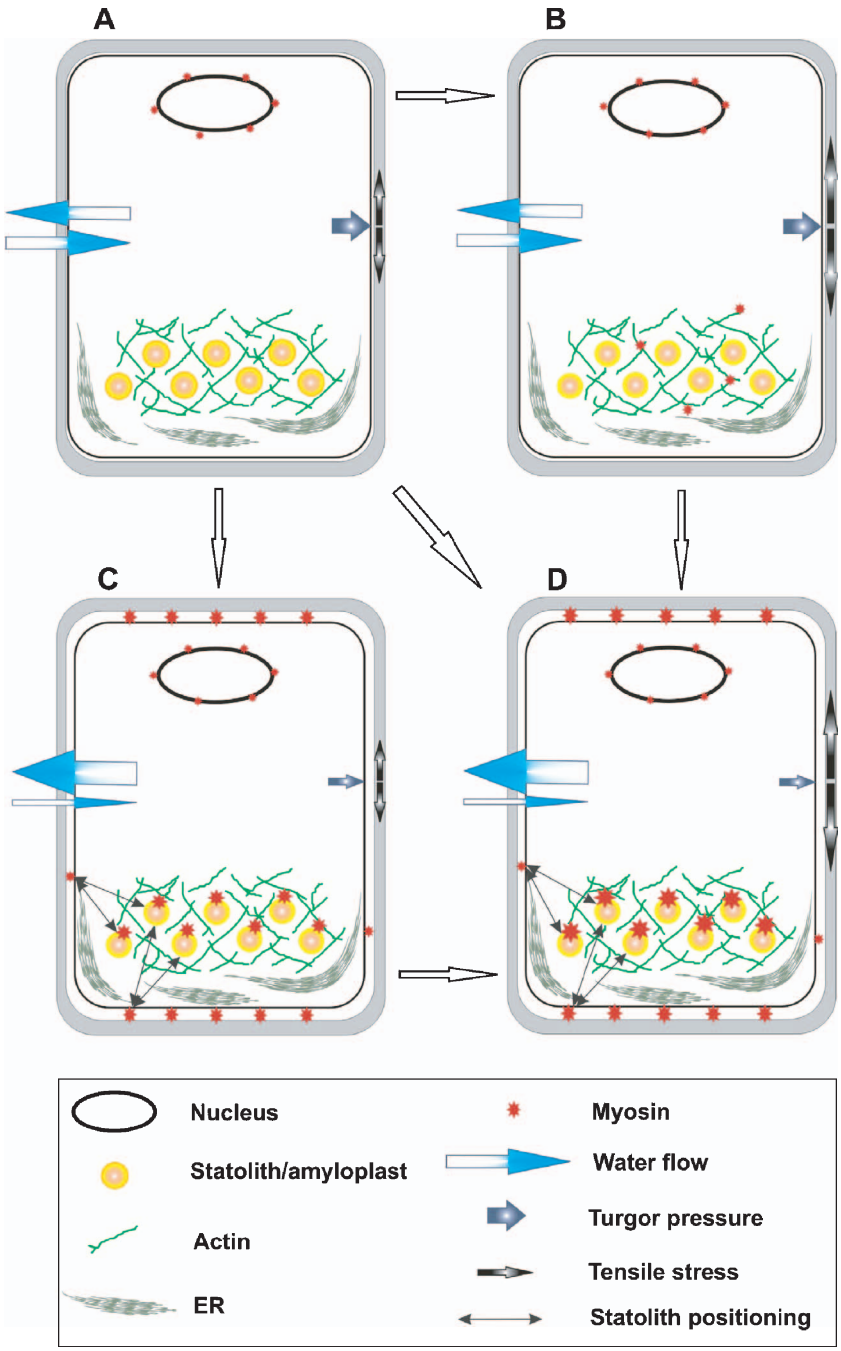

Fig. 7. A schematic model summarising the events observed in root cap cells during protoplast preparation. Under normal conditions (*A*), statoliths / amyloplasts are positioned at the apical part of the cell, enmeshed within a dense cytoskeletal network. Myosin VIII could be found, albeit in small quantities, mainly at the nucleus. The cell is under dynamic osmotic / water balance, and the resulting turgor pressure creates a certain level of tensile stress inside the surrounding walls. Application of hydrolytic enzymes alone (*B*) does not change osmotic balance, but induces progressive weakening of the walls, extent of which is dependent on the type of the enzyme used. This results in the increased tensile stress of the walls. After prolonged treatment myosin VIII labelling appears at statolith / amyloplast surfaces. Transfer of the roots into osmoticum alone (*C*), changes drastically water relations inside the cell. The protoplast shrinks due to increased relative water efflux. In effect, turgor pressure drops down, decreasing also the level of tensile stress of the walls. The cellular monitoring system detects shrinkage of the protoplast and changes in the relative positioning of the statoliths / amyloplasts. This induces rapid relocation of myosin VIII to organelle surfaces and, on a longer time-scale, also to plasmodesmata / adhesion foci at the walls to anchor actin filaments. When roots are subjected to digestion of cell walls (*D*), the above-mentioned phenomena add up as shrinkage of the protoplast is accompanied by the weakening of the mechanical stability of the walls. This intensifies the observed cellular responses without changing the characteristic time-dependent patterns, exemplified by the strength of myosin VIII labelling (the bigger the red star, the stronger labelling). Note that for the sake of clarity the model does not reflect precisely the subcellular localisation of myosin VIII.

gravisensing apparatus. To keep this machinery operative, the statoliths have to be repositioned in relation to plasma membrane and other organelles, and this again is done with the use of myosin VIII. We suggest that the signal transduction in those processes requires the existence of a functional WMC continuum. Somewhat similar conclusions, although without identification of the active element, have been drawn from experiments on *Ceratodon purpureus* protonemata cultivated in microgravity (Kern *et al*. 2001).

In conclusion, we stress the importance of the structural organisation of plant organs. Cells in roots or stems grow under defined mechanical environment and mechanical properties of epidermal cell are its major determinants (Hejnowicz *et al*. 2000). In our system, the external walls of the root are the last disrupted walls. Thus, the action of individual enzymes, although weakening the walls and affecting the individual components of plasma membrane–wall interface does not greatly change the cellular activities as long as the overall integrity of the organ is maintained. However, release of the cells, like for sloughing root-cap cells, frees them from their mechanical surrounding and immediately changes the myosin VIII location (Fig. 3). Similar experiments on suspension-cultured cells could reveal whether structural rearrangements parallel biochemical rearrangements observed previously (Aon *et al*. 1999).

Note added in proof: Myosin XI isoform MYAZ was recently found to localise to the surfaces of peroxisomes in an actin-dependent manner (Hashimoto *et al.* 2005). This finding suggests that besides mitochondria and plastids, myosins of class XI also move to peroxisomes.

Acknowledgments

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