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Dynamic rearrangements of transvacuolar strands in BY-2 cells imply a role of myosin in remodeling the plant actin cytoskeleton

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Summary. Plant cells typically contain a large central vacuole that confines the cytoplasm and organelles to the periphery of the cell and the vicinity of the nucleus. These two domains are often connected by transvacuolar strands (TVS), thin tubular structures that traverse the vacuole. The TVS are thought to act as important transport routes for the distribution of organelles and metabolites, and also to play a role in the positioning of the nucleus. Most TVS depend on internal actin filaments for their existence, and rearrangements of TVS can therefore indicate modifications in the actin cytoskeleton. In this study we describe time-lapse observations of tobacco BY-2 suspension-cultured cells that document the dynamic behavior of TVS. The TVS formed, branched, and collapsed, and their attachment points in the nuclear or cortical cytoplasm, as well as on other TVS, moved around. These dynamic rearrangements were inhibited within 5 min by the myosin inhibitor 2,3-butanedione monoxime (BDM). In particular, the movements of TVS attachment points and the variations in TVS length were significantly reduced in the presence of the drug. Similarly, movements of the nucleus were reduced by two thirds in BDM-treated cells. The number of TVS, together with the number of attachment and branch points, also dropped during BDM treatment. All effects of BDM on TVS dynamics were reversible upon removal of the drug. These results suggest a role for myosin motors in the rearrangement of TVS, which is likely to occur through their interaction with actin filaments.

Keywords: Transvacuolar strand; Cell architecture; 2,3-Butanedione monoxime; Myosin; Actin remodeling; Tobacco BY-2 suspension culture.

Abbreviations: BDM 2,3-butanedione monoxime; CAP cortical attachment point; NAP nuclear attachment point; TVS transvacuolar strand.

Introduction

Mature plant cells that are fully expanded typically contain a large central vacuole that occupies most of the intracellular space (Marty 1999). In these cells, the cytoplasm is relegated to a thin layer underneath the plasma membrane (cortical cytoplasm) and surrounding the nucleus (perinuclear cytoplasm). These two domains of cytoplasm are connected by thin tubular structures that traverse the central vacuole and are delimited by the tonoplast (see Fig. 1). These transvacuolar strands (TVS) are generally thought to serve two different functions. On the one hand, TVS provide a direct connection between the perinuclear cytoplasm and the cortical cytoplasm in distant parts of the cell, and as such, they can act as distribution routes for metabolites and organelles (Grolig and Pierson 2000). This function has been demonstrated for the secretory system, where it could be shown that Golgi stacks can move rapidly within TVS (Nebenführ et al. 1999). On the other hand, TVS are thought to be involved in anchoring the nucleus to a certain position within the cell (Williamson 1993). This role becomes particularly obvious in cells preparing for division as the nucleus moves into the future division plane (Katsuta et al. 1990; Kumagai and Hasezawa 2001).

The structural integrity of TVS depends on actin filaments that run inside these cytoplasmic tunnels. Treatment of plant cells with agents destabilizing filamentous actin (F-actin), such as profilin (Staiger et al. 1994, Valster et al. 1997), actin-depolymerizing factor (ADF1) (Hussey et al. 1998), cytochalasin B (Shimmen et al. 1995), or latrunculin B (van Gestel et al. 2002), leads to a rapid loss of TVS. The actin filaments within a TVS all have the same orientation and are bundled together by villin (Tominaga et al. 2000b). Microinjected antibodies against villin can disrupt the F-actin bundles in root hairs and thereby lead to broadening and eventual disappearance of TVS (Tominaga et al. 2000b). TVS can also be lost by microinjection of anti-calmodulin antibodies (Vos et al. 2000). In contrast, microtubules are

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present only in some TVS, with their presence mainly restricted to the G_2 phase of the cell cycle (Hasezawa et al. 1991, Kutsuna and Hasezawa 2002). TVS microtubules appear to be necessary for nuclear migration in tobacco BY-2 cells in preparation for cell division (Katsuta et al. 1990) but play little role in interphase TVS.

The different overall arrangements of TVS during the cell cycle have been described in great detail (Kumagai and Hasezawa 2001, Kutsuna and Hasezawa 2002). However, TVS are remarkably dynamic structures that continuously form, branch, and disappear within short timescales (Kamiya 1959, Salitz and Schmitz 1989, Shimmen et al. 1995). Since TVS depend on internal actin filaments for their maintenance, we speculate that these rapid TVS dynamics are likely brought about by rearrangements in the actin cytoskeleton. If this is the case, then TVS dynamics can be used as an easily observable substitute for actin filament remodeling. The mechanisms that mediate the rearrangements of actin cables in plants are only beginning to emerge (Deeks and Hussey 2003, Wasteneys and Galway 2003). In principle, two different possibilities are conceivable. On the one hand, these rearrangements could depend on the continuous formation and breakdown of new actin filaments. This kind of mechanism would likely depend on the concerted action of actin-nucleating or -branching proteins such as the ARP2/3 complex or formin, actin-bundling proteins such as villin or fimbrin, and actin-depolymerizing proteins such as profilin or ADF1 (Wasteneys and Galway 2003). On the other hand, the rearrangements of actin cables could also be brought about by the physical movement of existing actin cables. In this case, motor proteins that bind to actin filaments could be expected to translocate them along other actin filaments, or possibly cortical microtubules. These cytoskeletal motor-based actin rearrangements might, in principle, be compared to the role of kinesin motors in the organization of microtubules in trichomes or during cytokinesis (Liu and Lee 2001, Oppenheimer 1998). Of course, plant cells may also employ a combination of these two mechanisms to achieve reorganization of their actin cytoskeleton. In principle, it should be possible to distinguish the relative contributions of these two mechanisms to actin remodeling by inhibiting one of the pathways and observing the rearrangements of TVS (as a substitute for actin cables) at the same time.

Promising candidates for the postulated motor proteins that could mediate actin and TVS remodeling are myosins. Myosins are actin-based motor proteins that drive organellar movements and cytoplasmic streaming in plant cells (Reddy 2001). The motor activity of some myosins, among them a plant myosin from lily pollen tubes (Tominaga et al. 2000a), can be inhibited noncompetitively by 2,3-butanedione monoxime (BDM) (Herrmann et al. 1992). In several plant tissues, BDM also has been found to affect the organization of the actin cytoskeleton (Molchan et al. 2002, Tominaga et al. 2000a, van Gestel et al. 2002, Holweg et al. 2003); however, none of these studies has examined this effect in living cells.

In the course of a previous study on actomyosin-dependent Golgi stack movement (Nebenführ et al. 1999), we noticed that treatment of BY-2 cells with the myosin inhibitor BDM not only blocked Golgi stack movement but also seemed to affect the dynamics of TVS. In this study, we give a thorough description of TVS dynamics in living cells and quantify the effect of BDM treatment on their behavior. In particular, we find that BDM limits the motility of TVS and reduces their number.

Material and methods

Cell growth and maintenance

Transgenic tobacco BY-2 suspension cultures expressing a fluorescent Golgi marker (GmMan1-GFP) (Nebenführ et al. 1999) were grown at 27 °C in the dark in a modified Murashige–Skoog (MS) medium as described (Nagata et al. 1992). The Golgi marker was used to confirm that the BDM concentrations used were sufficient to inhibit directional organelle movements as described by Nebenführ et al. (1999) (data not shown). Actin filaments were visualized in a second transgenic BY-2 cell line stably expressing a talin-YFP construct. Briefly, a construct encoding a translational fusion between the actin-binding domain of mouse talin and yellow-fluorescent protein (Brandizzi et al. 2002) was introduced into BY-2 cells via *Agrobacterium tumefaciens*, as described in Nebenführ et al. (1999), except that selection for transformed cells occurred in the presence of 50 μg of hygromycin per ml. The talin-YFP construct was a kind gift from Drs. C. Hawes and F. Brandizzi (Oxford Brooks University, U.K.). The expression of either of the fluorescent markers did not appreciably change the morphology or physiology of the BY-2 cells. In particular, growth rates (as judged by culture density after 1 week) and intracellular dynamics were indistinguishable between untransformed and transformed lines.

Microscopy and drug treatment

3 to 4 days after transfer to fresh medium, suspension-cultured cells were placed on a round coverslip (25 mm diameter) treated with 0.1% polyethyleneimine to promote attachment of the cells to the glass surface. The coverslip was then placed in a custom perfusion chamber consisting of a second cover slip separated by an O-ring (Molecular Probes, Eugene, Oreg., U.S.A.; order number O14804) that acted as both a spacer between the cover slips and a seal. The assembled chamber was held together by a stainless steel frame. Fresh MS medium was perfused at 0.5 ml/min with the help of a peristaltic pump, by means of needles piercing the O-ring on opposite ends of the chamber. At the beginning of each experiment, BDM (Sigma) was freshly dissolved in an appropriate volume of MS to yield a final concentration of 40 mM. Lower concentrations (up to 30 mM) did not reproducibly lead to the dramatic responses described here. The supply of medium to the perfusion chamber could be switched between regular MS and MS supplemented with BDM at any time during the experiment.

A typical experiment consisted of three phases. During the first phase, cells were observed in regular MS for 5 min (pretreatment). For the second phase, the supply of medium was switched either to MS supplemented with BDM (drug treatment) or again to regular MS to serve as a control (mock treatment). After 15 or 20 min of drug or mock treatment, the supply of

medium was switched back to MS to wash out the drug and allow for recovery of the cells (recovery). Cells were observed under a Leica DM-RXA microscope (Leica Wetzlar, Federal Republic of Germany) with a 63× objective at differential interference contrast illumination. Images were captured at regular intervals as indicated with a digital camera (Sensicam; Cooke Corp., Tonawanda, N.Y., U.S.A.) and SlideBook software (Intelligent Imaging Innovations, Denver, Colo., U.S.A.) on a Macintosh computer (Apple Computer, Cupertino, Calif., U.S.A.). Actin filaments labeled with talin-YFP were visualized with a Leica SP2 confocal microscope at appropriate excitation and emission wavelengths.

Image analysis

Image processing and measurements were carried out in NIH Image, v. 1.62 (available at http://rsb.info.nih.gov/nih-image) as follows. First, we adjusted for subtle shifts of the cell during the observation period by manually placing the cell outline in register for successive images within a time-lapse series. This allowed us to compare precise X-Y positions of intracellular features from frame to frame and to calculate displacements in micrometers. The spatial calibration of the images was based on the resolution of the optical setup (0.106 μm/pixel). The number of various features (TVS, cortical [CAPs] and nuclear attachment points [NAPs], and branch points) was determined by counting them for every frame of the time-lapse sequence. TVS often leave the focal plane as they traverse the vacuole and therefore cannot be seen in their entire length. In these cases, only strands that were visible for at least 2 μm were counted. For the determination of movements, the centers of CAPs and NAPs were marked with the cross-hair tool of NIH Image and the coordinates recorded. For this analysis, only TVS that could be observed over their entire length were used. The position of the nucleus was determined as the center of a circle that was superimposed on the image to coincide with the nucleus. Images were processed for publication by the Photoshop application (Adobe Systems, San Jose, Calif., U.S.A.).

Results

Morphology and dynamics of transvacuolar strands

Since the BY-2 cell architecture changes dramatically during the cell cycle (Kutsuna and Hasezawa 2002), we restricted our observations to cells of an actively growing culture, each of which had a centrally suspended nucleus. On the basis of published data (Kutsuna and Hasezawa 2002), we assume that these cells were in either S or G_2 phase. TVS of these BY-2 cells can take on a variety of shapes when viewed with differential interference contrast optics (Fig. 1A). They range from very thin, straight connections that appear to be under tension to broader, slightly meandering channels that can contain larger organelles, presumably plastids. Every TVS has at least two ends to it, typically one at the cell cortex, the CAP, and the other at the nucleus, the NAP. Some TVS connect cortical cytoplasm to cortical cytoplasm and thus have two CAPs and no NAP. TVS often broaden ("fan out") near their ends, particularly at CAPs. In many instances, TVS split at a branch point somewhere along their length. The nucleus is often connected to the cortical cytoplasm by one, sometimes two thicker strands that are relatively immobile and may function in tethering this large organelle in the central region of the cell.

Observation of living BY-2 cells in a perfusion chamber with a continuous supply of fresh MS medium revealed that these transvacuolar strands were remarkably dynamic structures (time-lapse video sequences are available as electronic supplementary material at http://dx.doi.org/10.1007/s00709-004-0068-0). Among the dynamic behaviors were the movement of CAPs and the sliding of branch points (Fig. 1A). TVS seemed to appear and disappear rapidly in time-lapse video sequences and often could be detected for only a minute or less. These strands probably moved in and out of the focal plane between images, although some of them may also have formed de novo. Another consequence of the TVS movement was that the observed length of strands in the focal plane changed over time. In most cells, the position of the nucleus also shifted over time in a seemingly random fashion. While these observations were performed with transgenic cells expressing a fluorescent Golgi marker (GmMan1-GFP) (Nebenführ et al. 1999), similar dynamic behavior could also be observed in untransformed BY-2 cells (data not shown). However, the presence of the organelle marker allowed us to confirm the efficacy of the BDM treatment by observing the block of Golgi stack movements (Nebenführ et al. 1999).

BDM inhibits TVS dynamics

Cells treated with the drug BDM showed a dramatic decrease in TVS dynamics during the drug treatment (Fig. 1B; see video sequences). Individual cells were maintained for several minutes in normal MS medium (pretreatment), followed by 15 or 20 min in MS supplemented with 40 mM BDM (drug treatment), before a final wash in plain MS (recovery). As a control, we performed experiments with the same sequence of events, except that BDM was left out during the middle period (mock treatment). Qualitative observation of these cells revealed that BDM treatment led to a rapid reduction in the number of TVS and an apparent "rigid" state of the remaining strands (Fig. 1B). These changes were reversible within 10 to 20 min of removal of the drug (see supplementary video sequences at http://dx.doi.org/10.1007/s00709-004-0068-0).

We quantified the dynamic behavior of TVS in untreated and BDM-treated BY-2 cells to confirm these qualitative observations. The data presented here are derived from two cells that were observed at 30 s intervals. However, similar results could be obtained in all cells of the culture and also when cells were observed at 60 s intervals.

The number of TVS fluctuated in mock-treated cells, but remained relatively constant over the course of the experi-

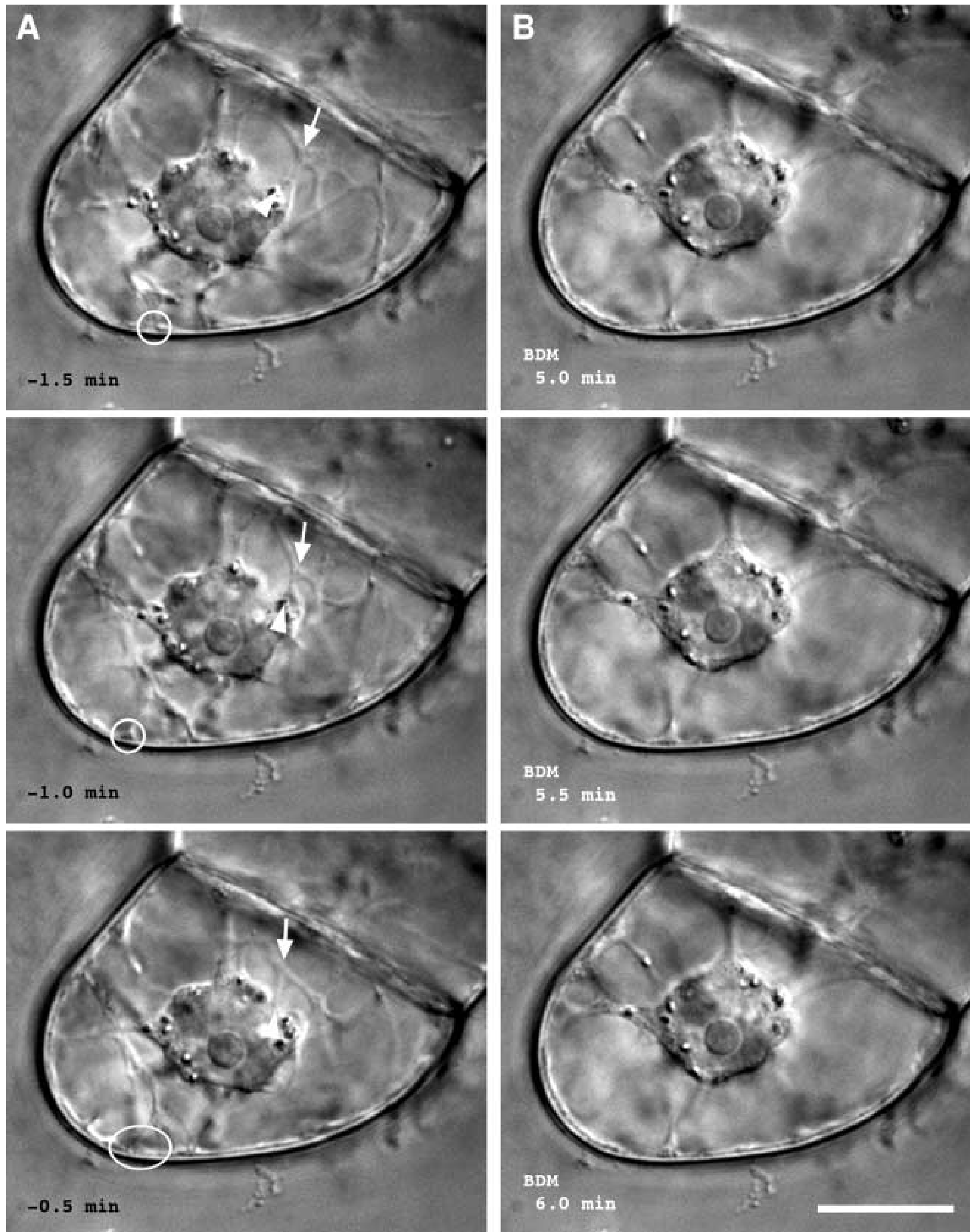

Fig. 1 A, B. Architecture of BY-2 cells and dynamic behavior of TVS are affected by BDM. Differential interference contrast images of a single BY-2 cell were taken at 30 s intervals, either during pretreatment (**A**) or in the presence of 40 mM BDM (**B**). The nucleus with its prominent nucleolus is clearly visible in the center of the cell. **A** Numerous TVS connect the nuclear to the cortical cytoplasm and show dynamic behavior in untreated cells. Arrows indicate a branch point that migrates along a TVS to the left. The associated NAP also moves to the left during the observation period (arrowheads). The circles enclose a CAP that first shifts to the left and then splits in two. **B** TVS appear rigid during BDM treatment. Dynamic rearrangements of TVS cannot be detected and a number of thinner, more branched TVS have disappeared, particularly in the upper right-hand and the lower left-hand areas of the cell. Bar: 10 μm

ment (e.g., 8.9 ± 0.2, mean with standard error, for "cell 2-mock" in Fig. 2A). In contrast, when the same cell was subjected to drug treatment, the number of TVS dropped significantly ($P < 0.01$; 2-tailed t-test) in the presence of BDM from 6.8 ± 0.4 to 3.5 ± 0.3 but recovered during the wash to 6.0 ± 0.3 (Fig. 2A, cell 2-BDM). A similar pattern, i.e., a reduction of the number of TVS by about 50%, was seen in another cell that had a larger number of TVS at the beginning of the experiment (Fig. 2A, cell 1-BDM). The average numbers of TVS for the different phases of the experi-

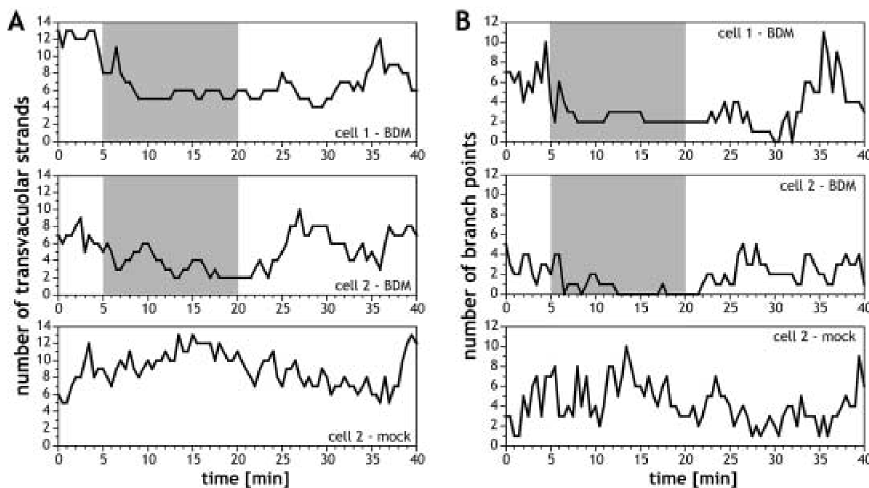

Fig. 2A, B. Number of TVS and branch points drops during BDM treatment but not during mock treatment. Cells were observed for the indicated period at 30 s intervals. Shaded areas indicate the presence of 40 mM BDM in the perfusion chamber. The number of TVS (**A**) and branch points (**B**) within the focal plane was determined for every video frame and plotted as a function of time. BDM treatment resulted in a lowering of the numbers and also an apparent reduction in variation. For "cell 2" both BDM and mock treatments are shown

Table 1. Drop of number of TVS during BDM treatment [a]

Treatment	Nr. of TVS in		
	Cell 1-BDM	Cell 2-BDM	Cell 2-mock
Pretreatment	12.3 ± 0.3	6.8 ± 0.4	7.8 ± 0.7
BDM or mock	5.5 ± 0.1	3.5 ± 0.3	10.7 ± 0.3
Recovery	7.4 ± 0.3	6.0 ± 0.3	7.8 ± 0.5

[a] The number of TVS in the focal plane was counted in every frame (30 s intervals) and then averaged for 11 (pretreatment), 24 (BDM or mock), or 22 (recovery) images. Data from the first few minutes of treatment and recovery were discarded, since the cells were transitioning from one state into the next (see Fig. 2). Values given are arithmetic means with standard errors of the mean

ment shown in Fig. 2 are summarized in Table 1. Thus, the number of TVS identifiable in the focal plane is reversibly reduced during the BDM treatment. A similar analysis was performed for the number of branch points (Fig. 2B), NAPs, and CAPs (data not shown). In every case, the number of observed instances of these points dropped during the BDM treatment and recovered during the washout.

A more direct way of quantifying the dynamic nature of TVS is to measure the displacement of CAPs, NAPs, or the nucleus over time. A technical problem with this approach is that many TVS can be seen for only one or a few frames, which makes accurate measurements of their movements impossible. Thus, we have focused our analysis on the more "stable" strands that could be observed for at least 5 frames (mean observation period, 9.5 ± 0.6 frames). It should be noted that this bias towards more static strands systematically underestimates the effect of BDM, since it ignores highly mobile TVS which are clearly absent in the presence of the drug. For each cell, at least three TVS connecting the nucleus and cortical cytoplasm were analyzed. Cells were observed at 30 s or 60 s intervals. Very similar numbers were found with both time intervals, suggesting that the movement of these TVS was not directional and that the periodicity of changes in movement direction is less than 30 s. Here, we only show data obtained from observations taken at 30 s intervals.

The average movement of the cortical and nuclear attachment points dropped from about 0.6 μm per frame during pretreatment to about 0.2 μm per frame in the presence of BDM (Fig. 3A). Thus, BDM limited the movement of TVS attachment points, even of relatively stable strands. The small displacements in the presence of BDM were near to the resolution limit of the optical setup used in the experiments. This pattern was also seen when the greatest displacement of an attachment point in a cell was compared under the two different conditions (Fig. 3B). Maximal displacements of NAPs and CAPs ranged from 0.5 to 0.8 μm per frame during BDM treatment but reached as far as 2.8 μm per frame for NAPs and 3.2 μm per frame for CAPs in untreated cells. This effect was most pronounced for CAPs, which displayed a higher mobility than NAPs in the absence of BDM. The reduced mobility of attachment points during BDM treatment also resulted in decreased length variation of TVS (data not shown), further confirming the rigid appearance of TVS in the presence of the drug.

The dynamics of TVS are also reflected in the mobility of the nucleus, since this organelle is tethered in space by several strands. Movement of the nucleus can therefore be seen as an integrated response that reflects the behavior of all TVS. In cells that were treated with 40 mM BDM, the average displacement of the nucleus between two

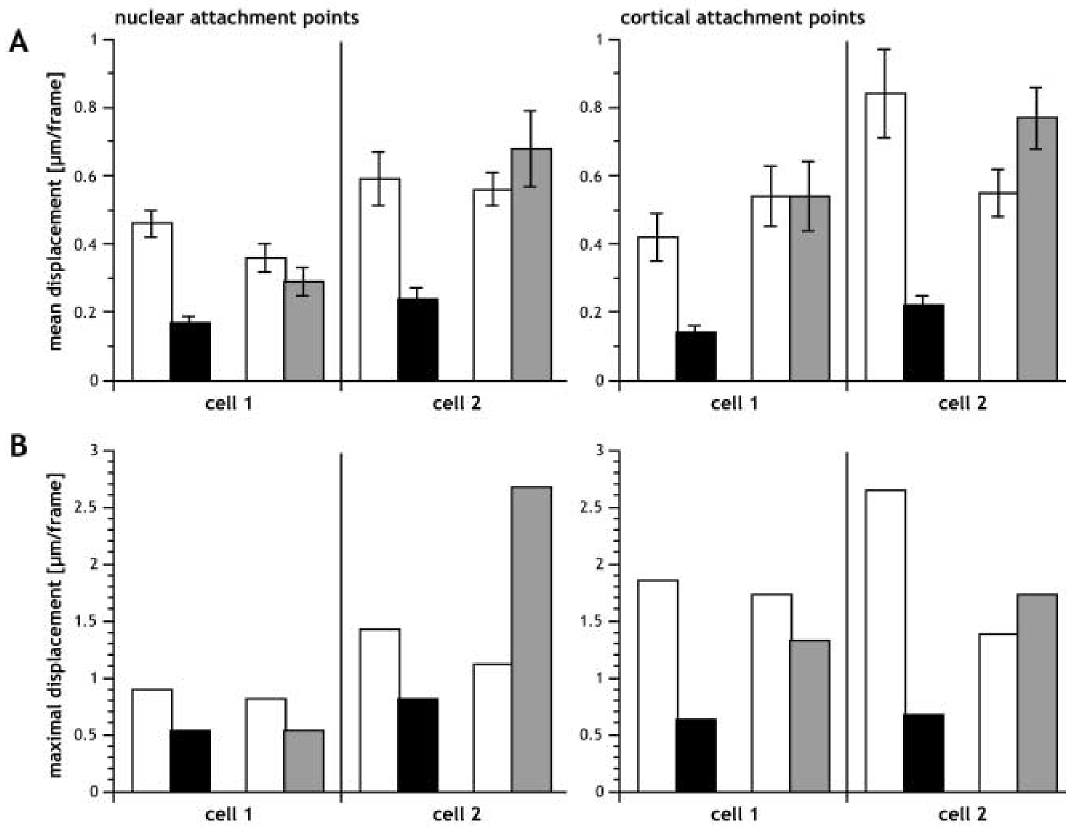

Fig. 3 A, B. Movements of TVS attachment points are reduced by BDM. The position of nuclear and cortical attachment points was determined for three TVS per cell, their frame-to-frame displacement calculated, and presented as either the average displacement (**A**) or the maximal displacement during the observation period (**B**). White bars indicate results from cells during pretreatment, black bars give the results from BDM treatment, and gray bars present data from mock treatment. Cells 1 and 2 are the same as in Fig. 2 and were imaged at 30 s intervals. Error bars indicate the standard error of the mean

subsequent frames dropped by 62% ± 6% (mean with standard error) (Fig. 4), whereas control cells showed only a minor change in activity during the observation period (reduction by 9% ± 6% during mock treatment; Fig. 4). In every case, the movement of the nucleus during BDM treatment was significantly less than that during pretreatment ($P < 0.01$, 2-tailed t-test).

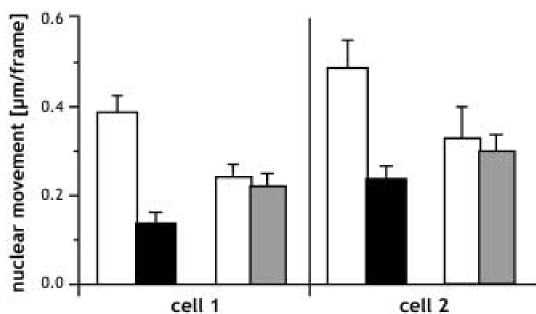

Fig. 4. Movement of the nucleus is reduced by BDM. The position of the nucleus was determined as the center of a circle that was superimposed on the differential interference contrast image to coincide with the nucleus. Frame-to-frame movement was calculated and averaged separately for pretreatment (white bars), drug treatment (black bars), and mock treatment (gray bars). Data are from the same cells as for Figs 2 and 3. Error bars indicate the standard error of the mean

BDM does not affect the integrity of actin filaments

The data presented so far demonstrate the dramatic effect of BDM on the dynamic behavior of TVS. In particular, we have shown that BDM limits the movement of attachment points and reduces the number of TVS. This reduction of TVS could result from a BDM-induced disintegration of actin filaments by nonspecific effects (also see the Discussion section). We investigated this possibility by visualizing actin filaments with talin-YFP fusion proteins (Brandizzi et al. 2002, Kost et al. 1998) in stably transformed BY-2 cells. In untreated cells, actin filaments form a dense mesh in the cortical cytoplasm (Fig. 5A), as well as surrounding the nucleus (Fig. 5B). TVS have prominent actin cables (Fig. 5B) that have been shown to be necessary for the maintenance of these strands (Staiger et al. 1994). During treatment of these cells with 40 mM BDM, actin filaments were still present in cortical and perinuclear cytoplasm, as

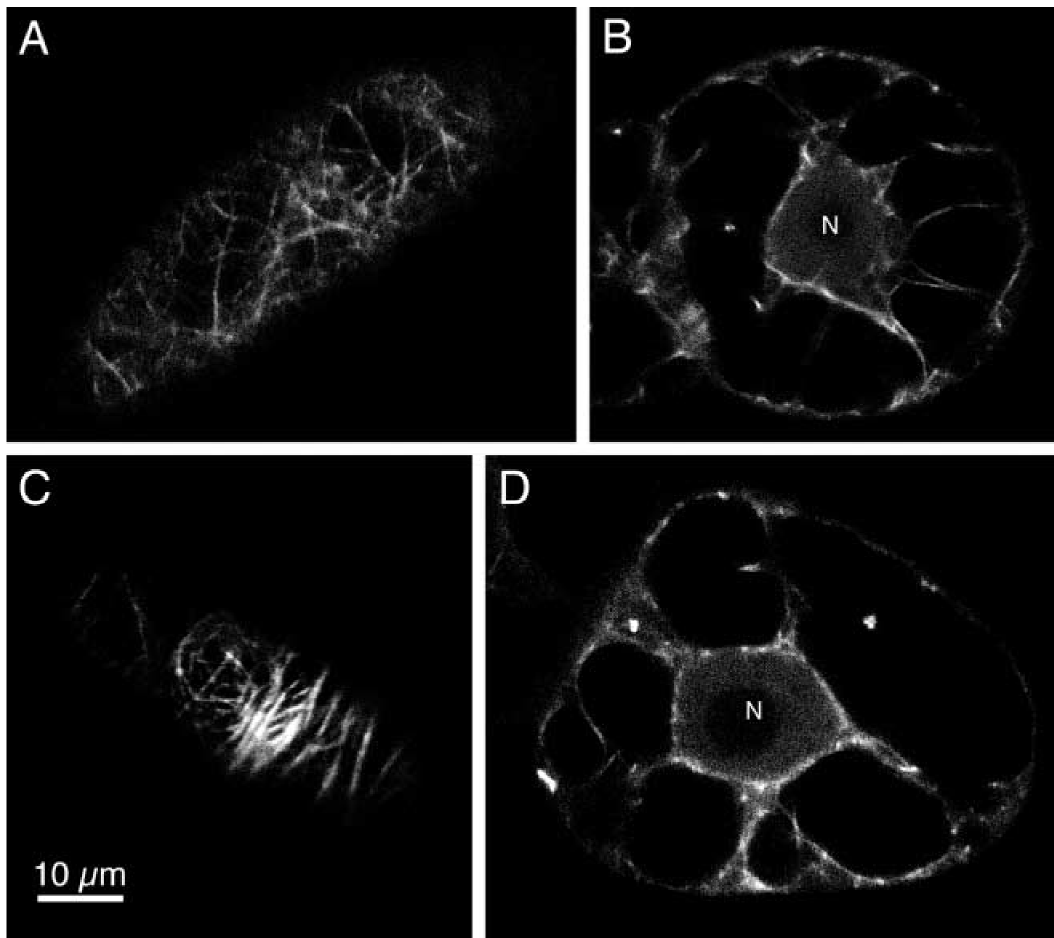

Fig. 5 A–D. BDM at 40 mM does not affect the stability of actin filaments. Actin filaments were visualized by confocal microscopy in BY-2 cells stably expressing a talin-YFP fusion construct. **A** Untreated cells display a fine actin network in the cortical cytoplasm. **B** Actin filaments are also visible around the nucleus (*N*) and in the TVS. **C** Cells treated with 40 mM BDM still contain a cortical actin network but often also show prominent transverse actin bundles. **D** Perinuclear and TVS actin filaments are still visible after 25 min in 40 mM BDM

well as in TVS (Fig. 5C, D). We conclude that 40 mM BDM does not lead to a disruption of actin filaments in BY-2 cells. However, in a number of treated cells we noticed prominent transverse actin cables in the cortical cytoplasm (Fig. 5C) that were not as pronounced in control cells, suggesting that BDM may affect the organization of actin filaments rather than their stability.

Discussion

TVS are remarkably dynamic structures

The TVS of the BY-2 cells observed in our experiments fall into two general categories. On the one hand, there are a few TVS that are fairly static over longer periods. These strands probably serve to anchor the nucleus in a specific position. On the other hand, there are many strands that are very dynamic and that can be observed in the differential interference contrast focal plane only for a short period. While some of these strands may form and then disappear again, many of them probably simply move in and out of the focal plane and do not disappear completely from the cell. While the most dramatic movements are obviously found in the second category, even the stable TVS can shift their position over time. The functional importance of these TVS movements is not known, but we assume that they support the even distribution of metabolites and organelles, similar to the cytoplasmic streaming also observed in these cells (Nebenführ et al. 1999). On a more speculative note, the continuous meanderings of the strands might be a way of searching the cellular space for signals that could induce a different cell architecture. Once a TVS encounters such a signal it may become stabilized in that position, which over time could lead to a relocation of the nucleus in that direc-

tion. Such a "search-and-capture" mechanism might be involved in nuclear positioning in preparation for asymmetric cell divisions (Scheres and Benfey 1999).

To our knowledge, this is the first attempt at a quantitative description of rapid TVS dynamics in plant cells. However, the continuous rearrangement of TVS has been known for a long time (Kamiya 1959) and has recently also been documented in impressive time-lapse movies (Cutler et al. 2000, movie available at http://www.pnas.org/cgi/content/full/97/7/3718/DC1). In this latter case, the TVS have been visualized not by differential interference contrast optics, but by fluorescent labeling of the tonoplast membrane that surrounds the TVS by means of an aquaporin-GFP fusion protein (Cutler et al. 2000). These data reveal that TVS dynamics are not restricted to BY-2 cells but also occur in other cell types, such as the hypocotyl epidermis of *Arabidopsis thaliana*.

Causes for the high degree of TVS mobility

There are no established theories that could explain the high level of motility observed for TVS. Given the dependence of the TVS integrity on actin filaments (Staiger et al. 1994), it is reasonable to assume that TVS dynamics are caused by rearrangements in their actin cytoskeleton. Thus, the TVS rearrangements could, in principle, be caused by two different mechanisms: one involving the growing and shrinking of actin filaments; the other, the action of motor proteins that move existing filaments around. For example, actin polymerization could lead to a lengthening of the filaments within the TVS. This should result either in a bending of the strand or, if an attachment point is not fixed, in a sliding of that point. Similarly, a shortening of actin filaments could result in increased tension and, hence, straightening of strands or sliding of one or both attachment points. The sliding of attachment points would probably require precisely regulated activation and inactivation of cross-linking proteins that anchor F-actin in the cortex or at the nucleus, respectively.

Alternatively, the movement of attachment points could result from an active displacement driven by motor proteins. This interpretation assumes that these motor proteins attach at or near the ends of the TVS F-actin and enable TVS movement along other fixed cytoskeletal elements, e.g., in the cortical cytoplasm. The movement of branch points may represent a case in which the ends of filaments move along the F-actin bundles of another strand. This model of motor-dependent movement of attachment points could also explain TVS branching, if we postulate that subsets of the actin filaments within a strand can be moved independently of each other. Taken to the extreme, this process could then result in the complete splitting of an existing strand, i.e., in the formation of new strands. Thus, this hypothesis predicts a role for motor proteins, possibly myosins, in the organization of the actin cytoskeleton (and, therefore, the TVS), similar to the role of kinesins in microtubule organization during trichome development or cytokinesis (Liu and Lee 2001, Oppenheimer 1998).

Effects of BDM and possible explanations

Clearly, simple observation of moving TVS does not distinguish between the above or other possible explanations. As a first step towards elucidating the mechanistic basis of TVS dynamics, we treated cells with the myosin inhibitor BDM, since myosins are likely candidates for the postulated motor proteins involved in actin reorganization. We observed greatly reduced activity of TVS in the presence of this drug. For example, the mobility of cortical attachment points was reduced to approximately one third of that in untreated cells. Similar results were obtained for nuclear movements and length variations of TVS, which were both significantly reduced in BDM-treated cells. Moreover, the number of TVS and, consequently, the number of nuclear and cortical attachment points dropped during the treatment period. Branch points, which tended to be highly dynamic in untreated cells, nearly disappeared in BDM-treated cells. In general, the cells seemed to enter a state of rigor in which all TVS movements were strongly curtailed.

While the overall effect of BDM on TVS dynamics appears clear, the interpretation of the underlying mechanisms is, unfortunately, not so straightforward. BDM has been described in the literature as a noncompetitive inhibitor of myosin activity (Herrmann et al. 1992) and the inhibitory effects of this drug have generally been interpreted as evidence for the involvement of myosin in a given process (Cramer and Mitchison 1995, Jedd and Chua 2002, Nebenführ et al. 1999, van Gestel et al. 2002, Holweg et al. 2003). However, BDM does not seem to affect all myosin classes equally (Ostap 2002). In the case of plant myosins, only one study has examined the effect of BDM on purified myosin (class XI from lily pollen tubes) and this found a 75% inhibition of actin-sliding activity at 40 mM BDM (Tominaga et al. 2000a). Thus, at least some plant myosins are sensitive to BDM, albeit only at relatively high concentrations (apparently above 10 to 20 mM; compare McCurdy [1999]). However, a recent report employing long-term exposure of tobacco VBI-0 suspension culture cells to 5 mM BDM revealed an inhibitory effect of this low concentration on cell division

activity (Holweg et al. 2003). Maybe different myosins respond to the drug with different affinities.

An additional complication of using BDM as a myosin inhibitor stems from the fact that this drug can affect other cellular processes. For example, it has been described as a chemical phosphatase (Fryer et al. 1988), and it can affect kinases, connexins, and K^+ and Ca^{2+} channels (Sellin and McArdle 1994); although, these effects have only been observed in animal cells. Nevertheless, given this plethora of alternative effects and the relatively high drug concentrations that have to be used for experiments, it is possible that the effects of BDM are not brought about solely by inhibition of myosin motor activity. Two particularly relevant concerns are possible effects of BDM on intracellular Ca^{2+} levels and on F-actin stability, since these effects may directly affect TVS. The first aspect was addressed in a recent study that directly measured the cytoplasmic Ca^{2+} concentration during BDM treatment and found no significant deviation from untreated cells (Molchan et al. 2002).

Several studies have examined the effect of BDM on the integrity and distribution of actin filaments. In pollen tubes and young root hairs, high BDM concentrations that inhibit cytoplasmic streaming (50 to 100 mM) resulted in a disorganized actin cytoskeleton but no apparent loss of actin filaments (Tominaga et al. 2000a). Interestingly, in the same study, the actin organization of older root hairs was not affected by BDM at concentrations of up to 100 mM (Tominaga et al. 2000a). Cells within the root tips of maize seedlings also displayed an altered distribution but no loss of actin filaments in the presence of low BDM concentrations (0.1 and 1 mM; Šamaj et al. 2000). Similarly, the actin filaments of *Tradescantia virginiana* stamen hair cells showed some rearrangement both in interphase and during cell division (30 mM BDM; Molchan et al. 2002). In soybean suspension cells, treatment with 10 mM BDM actually resulted in a stabilization of actin filaments (Grabsky et al. 1998). BDM treatment of cultured tobacco cells at moderate levels (20 mM) also did not lead to a loss of actin filaments but to a "disturbance of [transvacuolar] cytoplasmic strands" (van Gestel et al. 2002: p. 661). Finally, 20 mM BDM applied to cultured tobacco VBI-0 cells for 2 h did not disrupt cortical actin filaments but resulted in a "partial loss of subcortical actin bundles" (Holweg et al. 2003: p. 200). Combined with the results described here, it is reasonable to conclude that BDM does not directly affect the integrity of actin filaments in plant cells.

BDM seems instead to interfere with the regulation of the actin network, thus leading to an altered organization of filaments. We cannot exclude the possibility that a non-myosin-related effect of BDM is indirectly causing these changes. Nevertheless, the observed effects are certainly consistent with the idea that this disorganizing effect on the actin cytoskeleton is brought about by an inhibition of myosin motors (see also Šamaj et al. 2000). This interpretation has also been invoked to postulate that myosin motors provide part of the force for the proper expansion and alignment of the cell plate (Molchan et al. 2002). Our observations of TVS dynamics suggest that BDM leads to a reduced mobility and, thus, a stiffening of the actin cytoskeleton. This BDM-induced rigor is consistent with the BDM-induced increase in actin tension measured with optical tweezers in soybean suspension cultures (Grabsky et al. 1998).

In summary, TVS display a highly dynamic behavior that is sensitive to the myosin inhibitor BDM. The effects of the drug are consistent with a role of myosin motors in TVS motility and formation and thus indicate a role of actin-based motors in the remodeling of the plant actin cytoskeleton.

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