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Actin-filament-dependent remodeling of the vacuole in cultured mesophyll protoplasts

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Summary. The ability of plant cells to dedifferentiate represents an important survival strategy invoked in a range of situations from repair mechanisms following wounding to apomixis. Dedifferentiation requires that somatic cells reprogram and enter the cell division cycle. This in turn necessitates the accurate partitioning of nuclear content and organelles, such as chloroplasts, to daughter cells, thereby ensuring continuity of cellular information systems. The distribution of cytoplasm and its organelle content in mature plant cells is governed by a large, central vacuole, with connections between distant cortical and perinuclear cytoplasmic domains mediated by transvacuolar strands. Here we examined the changes to vacuolar architecture in *Arabidopsis thaliana* protoplasts expressing a green-fluorescent protein fusion to a δ-tonoplast-intrinsic protein (δTIP). We found that vacuolar architecture became increasingly intricate during protoplast culture with the development of numerous transvacuolar strands. The development of an intricate vacuolar architecture was an actin filament- and not microtubule-dependent process, as is the case in interphase plant cells. Furthermore, we show that myosin is required for this increased complexity of vacuolar architecture and the formation of subcortical actin filament arrays. Despite the likelihood that increased vacuolar invagination would allow better redistribution of cytoplasmic organelles, we found that repositioning of chloroplasts from cortical to perinuclear cytoplasm was not dependent on transvacuolar strands. Our findings indicate that the vacuole is a dynamic entity that develops a complex architecture before dedifferentiating plant cells enter cell division.

Keywords: Transvacuolar strand; *Arabidopsis thaliana*; Protoplast; Green-fluorescent protein; Dedifferentiation; Cytoskeleton; Organelle repositioning.

Abbreviations: AF actin filament; BDM 2,3-butanedione monoxime; GFP green-fluorescent protein; IVS internal vacuolar structure; LatB latrunculin B; MT microtubule; NEM N-ethylmaleimide; Ory oryzalin; TIP tonoplast-intrinsic protein; TVS transvacuolar strands.

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Introduction

Plants possess an exceptional capacity for regeneration by converting somatic cells into totipotent cells through the process of dedifferentiation. Dedifferentiation is important in a range of situations including repair mechanisms following wounding and during apomixis. Regeneration typically requires that cells dedifferentiate before entering into cell division. Nuclear inheritance during cell division is highly ordered, ensuring stringent, unbiased partitioning of chromosomes before cell division (Franklin and Cande 1999). Similarly, cytoplasmic components such as chloroplasts, mitochondria, and the endoplasmic reticulum display distinctive partitioning strategies that ensure unbiased inheritance before dedifferentiating cells enter cell division (Sheahan et al. 2004a).

The cytoplasm (and its organellar content) in a mature plant cell is confined to the subcortical and perinuclear regions by a large, central vacuole that occupies over 90% of cell volume (Reisen et al. 2005, Kutsuna and Hasezawa 2002). In combination with the cell wall, the central vacuole generates turgor that gives rigidity to the cell and drives plant growth. However, the plant vacuole is a dynamic and multifunctional organelle storing enzymes (Ono et al. 2006), ions, and metabolites (storage vacuoles), engulfing (autophagic vacuoles) and digesting (lytic vacuoles) cytoplasmic components, and being involved in cellular responses to environmental and biotic factors (for reviews, see Marty 1999, Bethke and Jones 2000). Specific aquaporins, known as tonoplast-intrinsic proteins (TIPs), act as tonoplast markers for these morphologically and functionally distinct vacuoles with αTIP found in autophagic

tonoplasts, γTIP in lytic tonoplasts, and δTIP in storage vacuoles (Jauh et al. 1999, Moriyasu et al. 2003).

Transvacuolar strands (TVS) are dynamic tubular structures that traverse the central vacuole, connecting cortical and perinuclear cytoplasmic domains. TVS continually migrate, branch, and re-form in the cell while concurrently permitting both molecules (Grolig and Pierson 2000) and organelles (Nebenführ et al. 1999) to redistribute between the perinuclear and cortical cytoplasm.

The development of the in vivo green-fluorescent protein (GFP) labeling technology (Chalfie et al. 1994) has enabled clear monitoring of vacuole dynamics in living cells (Hicks et al. 2004, Saito et al. 2005). However, to this point little was known about the dynamics of the vacuolar architecture during plant cell dedifferentiation, nor whether vacuole structure is required to mediate organelle redistribution before cell division. To address these questions, we examined the vacuolar architecture in cultured *Arabidopsis thaliana* mesophyll protoplasts expressing a GFP-δTIP fusion protein (Cutler et al. 2000). Single cells, such as protoplasts, that lack a cell wall are well suited to investigating such processes, as they provide cytological benefits not available to multicellular tissues. More importantly, the ability to induce protoplasts in culture to dedifferentiate with reasonable synchronicity provides a convenient system to analyse vacuolar dynamics in dedifferentiating cells.

Here we found that vacuolar volume in GFP-δTIP-expressing protoplasts increased rapidly during culture, almost twice as much as in nontransgenic protoplasts. The estimated surface area of the tonoplast initially decreased but subsequently increased to a level greater than the calculated surface area of the peripheral tonoplast alone, indicating increased lumenal vacuolar architecture. Indeed, protoplasts displayed an increasing prevalence of internal architecture during culture, particularly TVS, with a peak phase of remodeling occurring during the first 48 h of culture. Treatment with actin filament (AF)- and microtubule (MT)-disrupting agents indicated that the development of vacuolar architecture primarily required AFs, with little or no role for MTs. Treatments with various putative myosin inhibitors showed that maintenance of complex vacuolar architecture required myosin. Examination of AF structure in myosin inhibitor-treated cells showed that AFs traversing TVS were almost completely disrupted but that cortical AF arrays were less sensitive and possessed similarities to those of untreated cells. Interestingly, the clustering of chloroplasts around the nucleus, previously shown to be an AF-dependent process (Sheahan et al. 2004a), did not require the presence of TVS, indicating that chloroplast repositioning does not depend on AFs because of a requirement for AFs in TVS formation and maintenance.

Material and methods

Plant growth and protoplast isolation

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown horizontally in plates containing 0.5× Murashige and Skoog salts supplemented with 0.8% (w/v) agar and 1% (w/v) sucrose. Surface-sterilized seed were positioned on the agar surface and vernalized at 4 °C for 2 days before growing at 25 °C and a photoperiod of 16 h light and 8 h dark and fluence rate of 50 μmol of photons per m^2 per s. *Arabidopsis thaliana* mesophyll protoplasts were isolated from the aerial portions of 1- to 2-week-old plants and cultured as described by Sheahan et al. (2005). Axenic shoot cultures of tobacco were grown in culture pots containing 1× Murashige and Skoog salts supplemented with 0.8% (w/v) agar and 1% (w/v) sucrose. Axenic tobacco cultures were grown under the same conditions as *A. thaliana* and protoplasts isolated as described previously (Sheahan et al. 2005).

Labeling of tonoplast, AFs, and cytoplasm

The tonoplast was visualized by expression of a GFP fusion to *A. thaliana* δTIP (EGFP-δTIP [Cutler et al. 2000]) and AFs by a GFP fusion to the second actin-binding domain of the *A. thaliana* fimbrin, AtFIM1 (GFP-fABD2 [Sheahan et al. 2004b]). Protoplast cytoplasm was labeled by incubating protoplast suspensions with 1 mM fluorescein diacetate (Sigma, Sydney, Australia) for 5 min. Fluorescein diacetate was prepared as a 25 mM stock solution in 40% (v/v) acetone.

Inhibitor treatments

All inhibitors were obtained from Sigma, except oryzalin (Ory), which was from Crescent Chemical Co., Singapore). Latrunculin B (LatB), Ory, N-ethylmaleimide (NEM), and 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) were prepared respectively as 1 mM, 10 mM, 25 mM, and 50 mM stock solutions (1000×) in dimethyl sulfoxide. 2,3-Butanedione monoxime (BDM) was prepared fresh as a 1 M solution in sterile MilliQ water. Protoplast cultures were exposed to inhibitors throughout the culture period or for 25 μM NEM, 50 μM ML-7, and 15 mM BDM also for shorter durations as described in the text. Dimethyl sulfoxide (0.1% [v/v]) was used as a control in all experiments. Reversibility of myosin treatments was examined by removing with two washes in KM8p medium (Kao and Michayluk 1975).

Confocal imaging

Protoplasts withdrawn from culture at 24 h intervals were mounted in welled slides and examined with a confocal microscope (LSM510, Zeiss) equipped with a 40× C-Apochromat water-immersion objective. GFP expression or fluorescein diacetate labeling in protoplasts was visualized with a 488 nm argon laser and 500–530 nm band pass filter. Chloroplasts were viewed by autofluorescence using a 543 nm helium-neon laser and 650 nm long-pass filter.

Quantitative analysis of vacuolar changes

Quantitative data was obtained from at least three independent experiments (unless otherwise stated), with a minimum of 10 cells examined

in each replicate for each time point. Protoplast and vacuole volume were estimated from the radii (r) of cells by the formula that volume is equal to $\frac{4}{3}\pi r^3$, after fitting an ellipse to the midplane section of protoplasts and selecting the major axis as the radius. Surface area of the peripheral vacuole was estimated similarly, by the formula that surface area is equal to $4\pi r^2$ (see Fig. 1A). Changes to vacuolar architecture were quantitatively assessed from the amount of (a) internal lumenal vacuole structure, (b) TVS, or (c) the ratio of TVS to internal lumenal structure and by approximating tonoplast surface area. The prevalence of vacuolar architecture was determined by counting the proportion of cells in protoplast populations with internal lumenal vacuolar structure or with TVS. To approximate whole-cell, tonoplast surface area, serial z-sections (1 μm interval) of protoplasts were converted to 8-bit, grayscale image series. By the public domain NIH ImageJ 1.31v program (http://rsb.info.nih.gov/ij), images were calibrated, the cell periphery outlined with either the oval or polygonal selection tools, the background cleared, and a binary threshold applied. The "measure" command was then used to obtain tonoplast plan-area and bounding rectangle measurements for each slice of the whole-cell z-section. Results were exported to Microsoft Excel, by which the plan-area measurements were summed and peripheral tonoplast surface area calculated using half the maximum length of the bounding rectangle as the cell radius. The relationship between perinuclear chloroplast clustering and vacuolar architecture was assessed by determining the proportion of cells with observable TVS in cells with perinuclearly positioned chloroplasts.

Results

Vacuole expands and tonoplast surface area amplifies during protoplast culture

During culture, protoplast volume increased (Fig. 1B). Considering that the vacuole occupies over 90% of the volume in mature plant cells, we hypothesized this increase was due to vacuolar expansion. Estimating the volume of the vacuole from its diameter at the cell mid-plane in near-spherical cells (Fig. 1A, B) demonstrated that the vacuole expanded during the first 96 h of protoplast culture, with the most rapid expansion occurring between 48 and 72 h, after which time the increase stabilized (Fig. 1B). Intriguingly, this expansion was, on average, 1.8-fold greater than the increase in protoplast volume observed in nontransgenic *A. thaliana* protoplasts. It is probable that this effect was an artifact of over-expressing the GFP-δTIP fusion protein (see Reisen et al. 2003).

The total surface area of the tonoplast was approximated by summing the plan-area of tonoplast membrane in op-

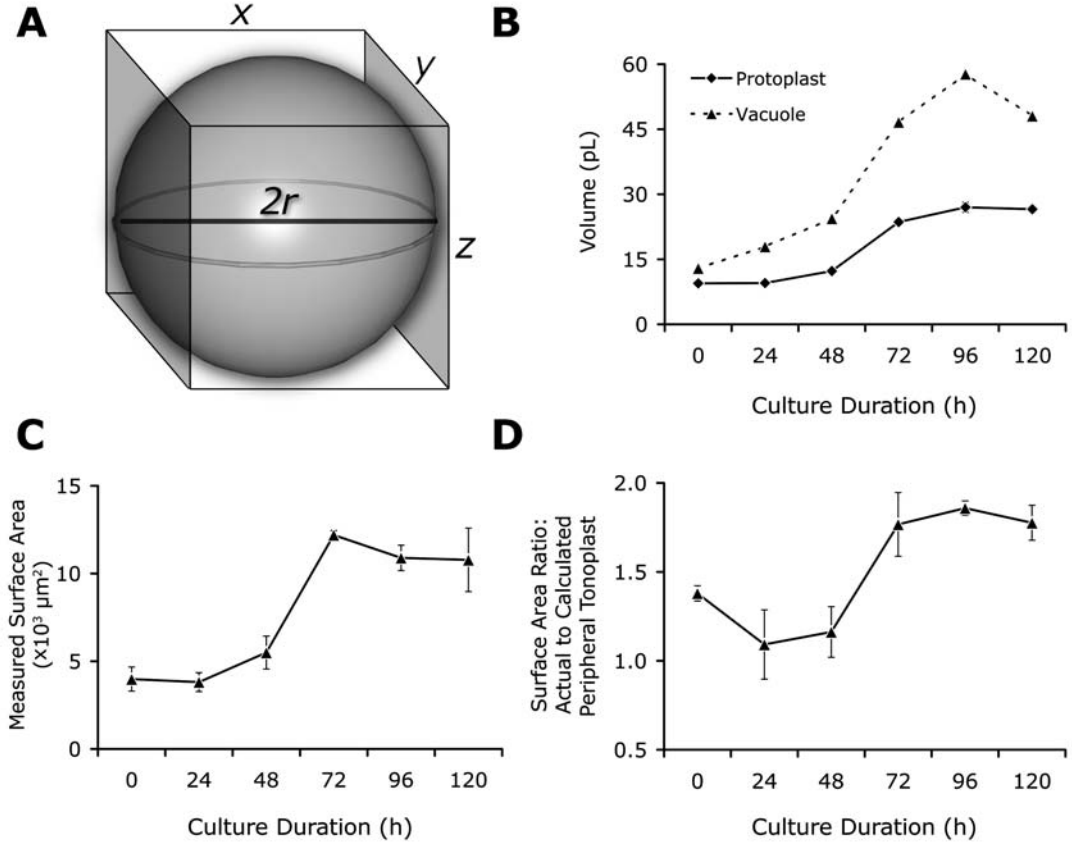

Fig. 1 A–D. Vacuole expands and tonoplast surface area amplifies during protoplast culture. **A** Schematic diagram indicating how the midplane diameter (2r) in protoplasts or vacuoles was measured for the calculation of protoplast and vacuole volume or peripheral vacuolar surface area. **B** Calculated protoplast and vacuolar volumes; **C** measured tonoplast surface area per cell; **D** ratio of the actual measured tonoplast surface area to that calculated for the peripheral tonoplast from the midplane diameter of vacuoles

tical sections taken through the whole protoplast. This analysis indicated that during the initial 24 h of protoplast culture, the amount of tonoplast surface area changed little. However, subsequent to this, tonoplast surface area increased substantially, with the greatest rate of increase occurring between 48 and 72 h of protoplast culture, and plateauing thereafter (Fig. 1C). As a measure of internal vacuolar structure (IVS), we computed the ratio of the measured tonoplast surface area to the calculated surface area of the peripheral tonoplast (SA_M-to-SA_C ratio). Interestingly, within the first 24 h of culture the ratio decreased, but thereafter increased, until 72 h of culture, where it

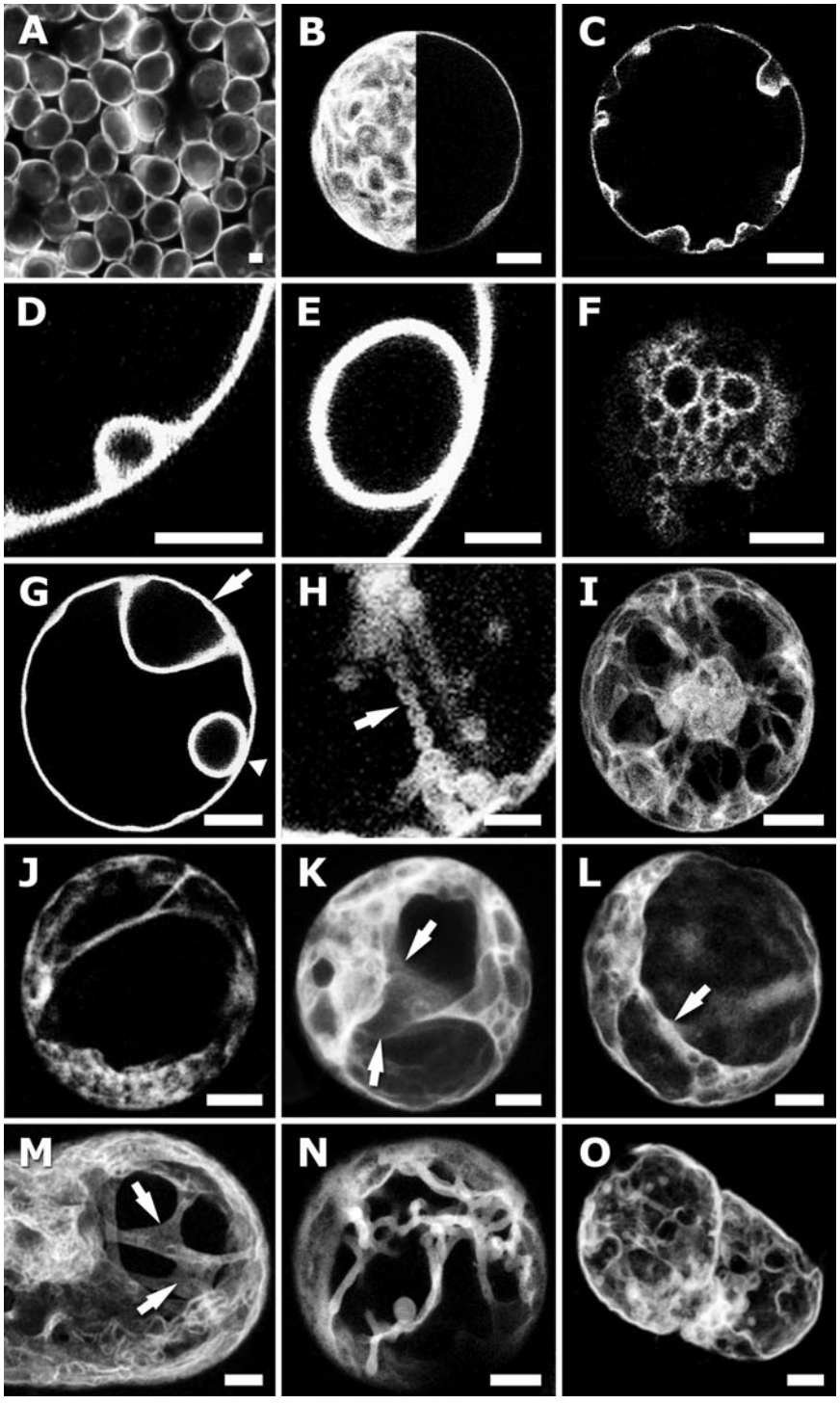

Fig. 2 A–O. Changes to vacuolar architecture during protoplast culture. Tonoplast visualized by confocal microscopy in *A. thaliana* protoplasts expressing a GFP-δTIP fusion protein. **A** and **B** Tonoplast structure in a 25 μm deep section of mesophyll tissue (**A**) and a freshly isolated protoplast (**B**). **C** Membranous protrusions into the cell. **D** and **E** Small (**D**) and large vesicles (**E**) interior to the peripheral tonoplast. **F** Clustering of vesicles. **G** Vesicles that appear continuous (arrow) and discontinuous (arrowhead) with the peripheral tonoplast. **H** String of vesicles (arrow) that will perhaps coalesce to form a tubular structure. **I** TVS connecting perinuclear and cortical cytoplasmic domains. **J** and **K** Example of a fine TVS (**J**) and tonoplast sheet (**K**). Arrows in **K** define a 17.5 μm wide segment. **L** A TVS (arrow) connecting two regions of cortical cytoplasm. **M** Branch connections (arrows) between TVS. **N** A labyrinthine vacuolar network. **O** Vacuolar architecture in a divided protoplast. Images are confocal projections of partial cells (A, left half of B, I–O) or single optical sections (right half of B, C–H). Bars: A–C, G, and I–O, 10 μm; D–F and H, 5 μm

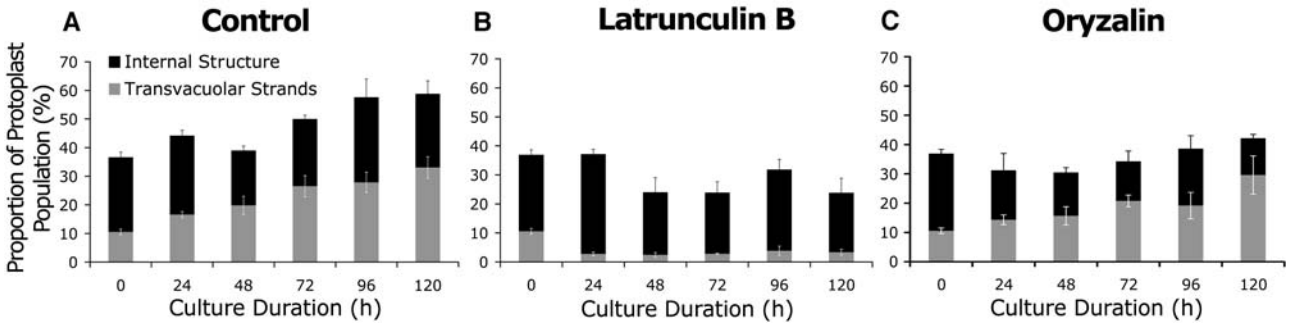

Fig. 3 A–C. Quantification of change to vacuolar architecture during protoplast culture in control and cytoskeletal-inhibitor-treated cells. The proportion of cells possessing IVS (black bars) and TVS (grey bars) was determined during protoplast culture for controls (**A**), LatB-treated (**B**), and Ory-treated cells (**C**). The proportion of cells with IVS increased during culture, as did cells with TVS. **A** The proportion of cells with IVS that possessed TVS also increased. **B** The proportion of cells with IVS in LatB-treated cultures remained relatively constant while the number of cells with TVS decreased significantly. **C** Ory-treated cells showed a trend similar to that for controls, albeit at reduced levels. Cells with TVS are a subset of those cells with IVS

stabilized (Fig. 1D). This finding indicates that the proportion of the surface area of the tonoplast interior increases relative to the peripheral tonoplast during culture (due to the formation of IVS) and that the vacuolar architecture remodels during early protoplast culture.

Remodeling of vacuolar architecture before protoplasts divide

In fresh protoplast preparations (time zero of protoplast culture), the GFP-δTIP fusion protein was predominantly localized at the cell periphery (Fig. 2B). The complexity of vacuolar architecture in these cells was limited, similar to that observed in immature mesophyll cells of intact *A. thaliana* seedlings (Fig. 2A and see supplementary video 1 available at http://dx.doi.org/10.1007/s00709-006-0236-5). This limited complexity was in contrast to other cell types, for example, epidermal cells of the hypocotyl, which frequently contained many, highly dynamic TVS per cell (see supplementary video 2 at the web page indicated above). The vacuoles of freshly prepared protoplasts, however, were not entirely devoid of structure. All cells that displayed the vacuolar architectures more intricate than a simple peripheral tonoplast (which includes invaginations due to cytoplasmic organelles such as chloroplasts and the nucleus) were defined as having IVS. In fresh protoplast preparations, approximately one-third of cells displayed IVS (Fig. 3A). Tonoplast protrusions from the cell periphery into the vacuole were common in such cells (Fig. 2C),

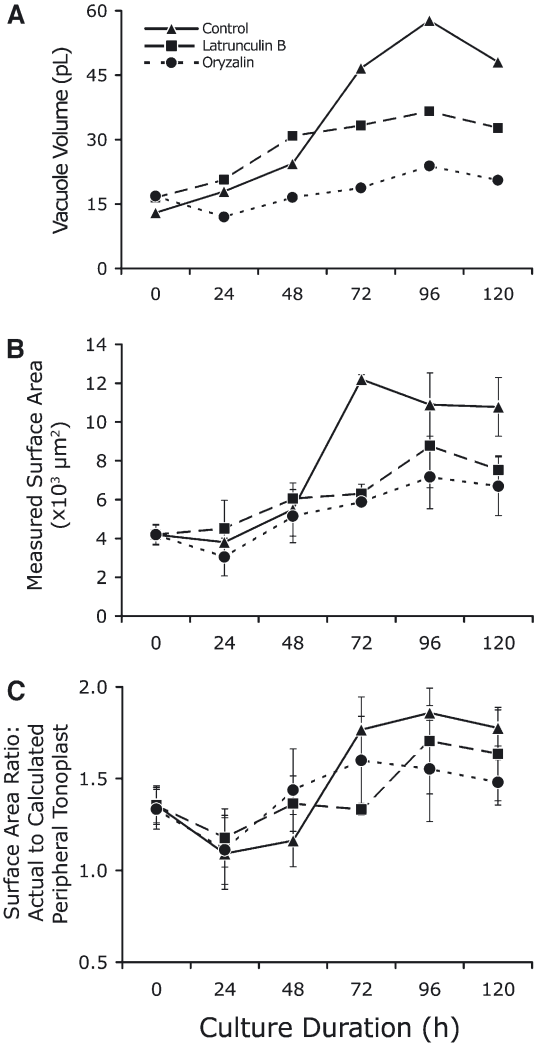

Fig. 4 A–C. Quantification of change to vacuole volume and surface area during protoplast culture in cytoskeletal-inhibitor-treated cells. **A** Calculated vacuole volumes; **B** measured tonoplast surface area per cell; **C** ratio of actual measured tonoplast surface area to that calculated for the peripheral tonoplast from the midplane diameter of vacuoles. Both vacuole volume and measured tonoplast surface were smaller in LatB- and Ory-treated cells compared to controls. Measured surface area to calculated peripheral tonoplast surface area ratios were not significantly different between controls and inhibitor-treated cells

as were small vesicle-like bodies that ranged in diameter from 2 to 10 μm (Fig. 2D, E). Such vesicle-like bodies frequently tended to organize into clusters along the interior of the peripheral tonoplast (Fig. 2F) and often appeared at least partially continuous with the peripheral tonoplast (Fig. 2G). Occasionally, vesicle-like bodies appeared to be coalescing into more substantive tubular structures (Fig. 2H).

During protoplast culture, the vacuole developed an increasingly complicated architecture. The appearance of numerous TVS during protoplast culture created a reticulate vacuolar morphology (for example, see Fig. 2I, M–O). Meandering TVS formed an intricate network of cytoplasmic channels, while other TVS, better classified as tonoplast sheets, transected large portions of cytoplasm (Fig. 2K). TVS varied in width from fine tubules, 0.5 μm in diameter (Fig. 2J), to wide sheets, over 15 μm at their widest points (Fig. 2K). TVS usually connected cortical and perinuclear cytoplasmic domains (Fig. 2I) and were particularly evident during the later phase of protoplast culture (72 h onward); however, cortex-to-cortex TVS (Fig. 2L), branched TVS (Fig. 2M), and labyrinthine networks (Fig. 2N) were also common. TVS invariably flanged out at their ends and at points of juncture. The prevalence of TVS increased relatively linearly with protoplast culture. In fresh protoplast preparations, only 10% ± 3% (mean with standard error, n = 5) of cells possessed observable TVS, but by 120 h of culture, this value increased to 30% ± 5% (n = 5) of cells (Fig. 3A). Further, in the subpopulation of cells possessing IVS, the ratio of TVS to IVS increased approximately 2-fold during protoplast culture (Fig. 3A), suggesting that the IVS is converted into functional TVS. In divided cells, TVS organized into complex arrays (Fig. 2O) that typically consisted of radial TVS emanating from daughter nuclei and extending to the cell cortex.

Establishment of intricate vacuolar architecture requires actin but not microtubule cytoskeleton

In interphase plant cells, AFs are required for the formation of TVS, with MTs required for nuclear centering during specific phases of the cell cycle (Kutsuna and Hasezawa 2002). We investigated the role of AFs and MTs in the establishment of vacuolar architecture during protoplast culture by treating protoplasts with the AF-disrupting agent LatB (1 μM) or the MT-disrupting agent Ory (10 μM). Quantification of the number of cells with IVS in LatB- and Ory-treated cultures demonstrated that AFs and MTs were not crucial for maintaining IVS, but were important for the formation of new vacuolar architecture (Fig. 3A–C). Accordingly, there was a reduced increase in vacuole volume and tonoplast surface area in LatB- and Ory-treated protoplasts compared with controls (Fig. 4A, B). Although vacuole expansion and amplification of tonoplast surface area was reduced, the SA_M-to-SA_C ratio increased during culture, indicating that relative to volume, there was some level of tonoplast amplification in inhibitor-treated cells (Fig. 4C).

Treatment with LatB, but not Ory, clearly reduced the prevalence of protoplasts with TVS, indicating that the maintenance and formation of new TVS was an AF- but not MT-dependent process (Fig. 3B, C). Indeed, although the

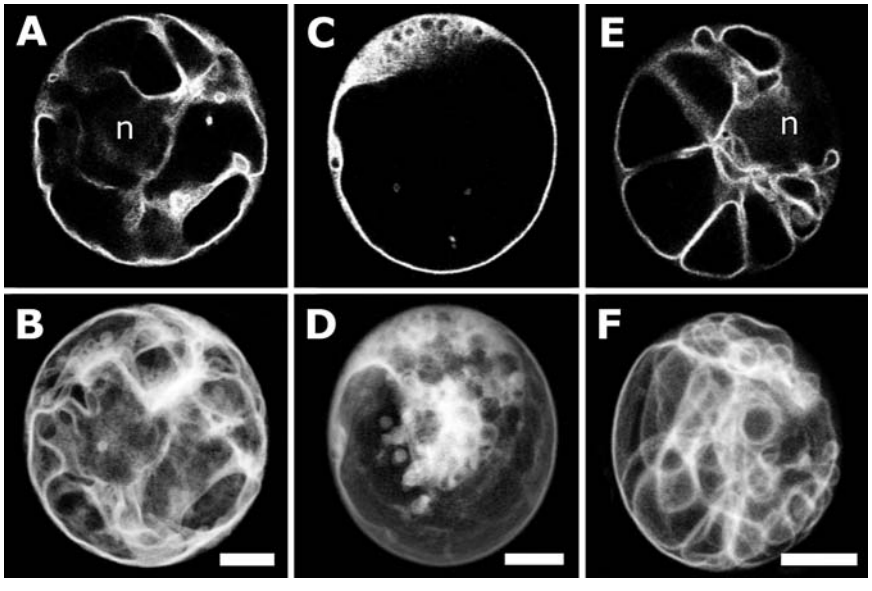

Fig. 5 A–F. Vacuolar architecture in control cells and cells treated with cytoskeletal inhibitors. Representative cells after 72 h of protoplast culture for control (**A** and **B**), LatB-treated (**C** and **D**), and Ory-treated cells (**E** and **F**). **A** Intricate vacuolar architecture and centered nuclei (*n*) were prevalent in controls after 72 h of culture. **B** LatB-treated cells rarely contained TVS or intricate vacuolar architecture. **C** Ory-treated cells often possessed TVS and intricate vacuolar architecture, but nuclei (*n*) were not centered. Images are single midplane optical sections (A, C, E) or confocal projections of partial cells (B, D, F). Bars: 10 μm

proportion of cells with IVS remained relatively constant in LatB-treated cultures, the proportion of those cells with TVS decreased, indicating that IVS was unable to convert into TVS. Disrupting AFs or MTs, however, did not appear to stop the phase of vacuolar remodeling that occurred in early protoplast culture as the measured tonoplast surface area, relative to the calculated surface area of the peripheral tonoplast, showed changes similar to controls (Fig. 4C).

Clear changes to vacuole architecture caused by application of LatB or Ory were visible after 72 h of protoplast culture. Control cells commonly possessed a centralized nucleus and several cortex-to-perinuclear TVS (Fig. 5A, B). LatB-treated cells had either vesicle-like IVS and no TVS (Fig. 5C, D) or no IVS at all, while Ory-treated cells had transvacuolar architecture similar to controls, but not centralized nuclei (Fig. 5E, F).

Requirement for myosin in maintenance of vacuolar and actin cytoskeleton architecture

TVS are highly dynamic structures (Shimmen et al. 1995, Hoffman and Nebenführ 2004) that continually form, branch, and disappear in the cell. Hoffmann and Nebenführ (2004) assessed TVS dynamics in actively growing BY-2 cells and found that the chemical phosphatase BDM stopped TVS dynamics but also reduced the number of TVS. Here, we investigated the role of myosins in the maintenance and formation of new TVS during protoplast dedifferentiation by application of BDM (15 mM), the

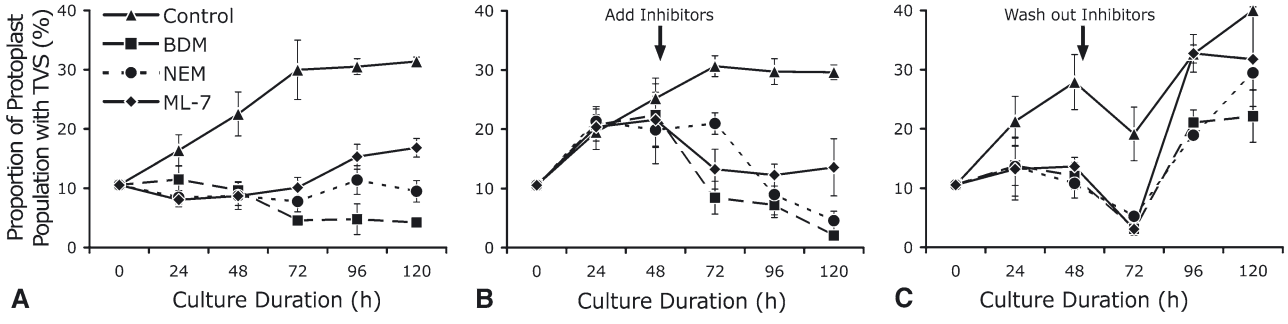

Fig. 6 A–C. Quantification of change to vacuolar architecture during protoplast culture in cells treated with putative myosin inhibitors. **A** Proportion of cells with TVS during protoplast culture when myosin inhibitors were added at the initiation of culture; **B** inhibitors added after 48 h of protoplast culture; **C** inhibitors washed out after 48 h of culture. Addition of myosin inhibitors decreased the prevalence of cells with TVS when added at the initiation of protoplast culture. Addition of myosin inhibitors after 48 h of culture also decreased TVS prevalence in the protoplast population, indicating a requirement for myosin in maintenance of transvacuolar architecture. Removal of myosin inhibitors increased the prevalence of TVS in the protoplast population, indicating a reversible inhibition of TVS formation. Taken together, theses results indicate that myosin plays a role in the establishment and maintenance of transvacuolar architecture. Values are means with standard errors from all measurements as in Table 1

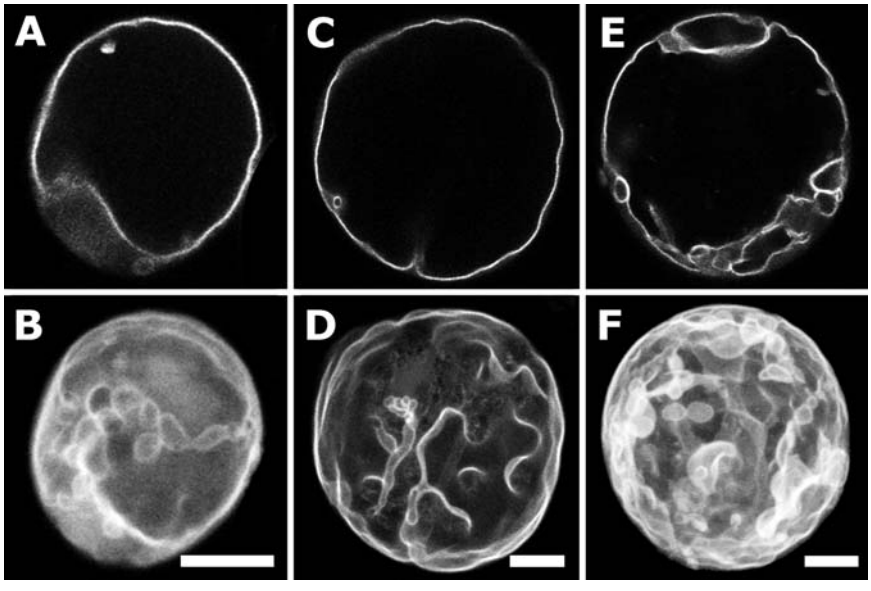

Fig. 7 A–F. Vacuolar architecture in cells treated with putative myosin inhibitors. Representative cells after 72 h of protoplast culture for BDM-treated (**A** and **B**), NEM-treated (**C** and **D**), and ML-7-treated cells (**E** and **F**). In all treatments, vacuolar architecture was altered with a significantly decreased prevalence of TVS. Images are single midplane optical sections (A, C, and E) or confocal projections of partial cells (B, D, and F). Bars: 10 μm

sulfhydryl-alkylating agent NEM (25 μM), or the myosin light-chain kinase inhibitor ML-7 (50 μM) (Saitoh et al. 1987), all of which have been used previously to inhibit myosin-based activities in plant cells (for examples, see Liebe and Quader 1994, Liebe and Menzel 1995, Nebenführ et al. 1999, Molchan et al. 2002).

Similar to LatB treatment, application of BDM, NEM, or ML-7 decreased the prevalence of TVS in protoplasts, suggesting that there is a requirement for myosin in the establishment of new TVS (Fig. 6A). Furthermore, applying the inhibitors to protoplasts after 48 h of culture, when many TVS had already formed, decreased the prevalence of TVS, indicating that myosin is likely to be required for the maintenance of TVS (Fig. 6B). These observations were not simply the result of decreased cell viability, as removal of the inhibitors allowed TVS to re-form (Fig. 6C). Treatment with BDM produced the most severe effect to transvacuolar architecture, with TVS rare in protoplasts cultured in the presence of the inhibitor for 72 h (Fig. 7A, B). Vacuolar structure in the cells was restricted to vesicle-like bodies and short tubular sections of tonoplast. Results were similar, although less severe, in protoplasts treated with NEM (Fig. 7C, D) or ML-7 (Fig. 7E, F). Taken together, these results indicate that myosin is likely to play a role not only in TVS dynamics but also in the formation and maintenance of transvacuolar architecture.

To determine if the inhibitors of myosin-based activity were causing depolymerization of the actin cytoskeleton, we examined AF organization in inhibitor-treated protoplasts expressing GFP-fABD2 (Sheahan et al. 2004b). In control cells after 72 h of culture, dense cortical AF arrays and AF bundles that traversed the subcortical cytoplasm were present (Fig. 8A, B). In contrast, LatB-treated cells showed completely depolymerized cortical and subcortical actin arrays, with the GFP-fABD2 fusion protein localizing to beads of fluorescence (Fig. 8C, D). Treatment with BDM (Fig. 8E, F), NEM (Fig. 8G, H), or ML-7 (Fig. 8I, J) caused an effect intermediate between those shown by control and LatB-treated cells. Cortical AFs in these cells remained reasonably intact, although the density of AFs was frequently much lower than in controls. Subcortical AF arrays in these cells, however, were no-

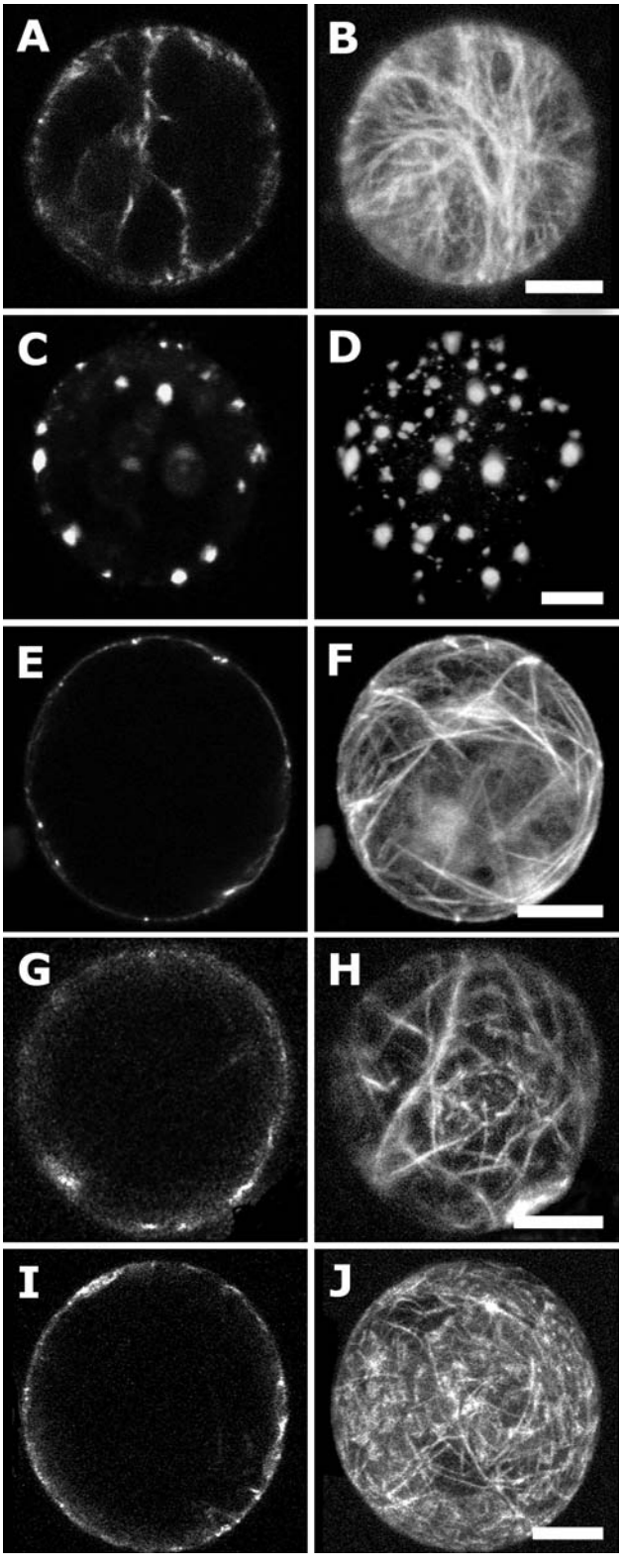

Fig. 8 A–J. Actin cytoskeleton structure in control and inhibitor-treated cells. The actin cytoskeleton visualized by GFP-fABD2 expression in protoplasts after 72 h of culture. **A** and **B** Controls; **C** and **D** LatB-treated cells; **E** and **F** BDM-treated cells; **G** and **H** NEM-treated cells; **I** and **J** ML-7-treated cells. The actin cytoskeleton in control cells was composed of a fine cortical array of AFs and bundles of AFs in TVS. LatB-treated cells typically possessed completely disrupted AF networks. AF bundles in TVS were rare in BDM-, NEM-, and ML-7-treated cells, which had varying degrees of disruption to the cortical AF array. Images are single midplane optical sections (A, C, E, G, and I) or confocal projections of partial cells (B, D, F, H, and J). Bars: 10 μm

Table 1. Actin cytoskeleton organization in inhibitor-treated cells[a]

Treatment	% of protoplast population with:		
	TVS	Cortical AFs only	Actin cytoskeleton completely depolymerized
DMSO (0.1%, v/v)	35 ± 3.2	63 ± 3.0	2 ± 0.4
LatB (1 μM)	2 ± 0.4	10 ± 2.3	88 ± 2.7
BDM (15 mM)	4 ± 1.3	43 ± 3.9	53 ± 4.8
NEM (25 μM)	5 ± 0.6	54 ± 2.5	41 ± 2.4
ML-7 (50 μM)	8 ± 1.7	60 ± 3.1	32 ± 1.9

[a] GFP-fABD2-expressing cells were treated with the inhibitors indicated from the onset of protoplast culture. The proportion of the protoplast population possessing TVS, cortical AFs only, or a completely depolymerized actin cytoskeleton was determined after 72 h culture. Values are means with standard errors, n = 2, with 300 cells counted in each replicate experiment

tably absent (Fig. 8E, G, I). Quantitative analysis of cells treated with BDM, NEM, or ML-7 indicated that all had the potential to cause complete depolymerization of the actin cytoskeleton (Table 1). These findings suggest that there may be a differential sensitivity of cortical and subcortical AFs to these putative myosin-disrupting agents.

Although no label for the tonoplast expressed in tobacco was available at the time of this study, we were able to observe similar changes to vacuolar architecture in tobacco mesophyll protoplasts by labeling the cytoplasm with fluorescein diacetate. Similar to *A. thaliana*, during the culture of tobacco mesophyll protoplasts, there was an AF- and myosin-dependent increase in the number of TVS (data not shown).

Development of TVS is not essential for chloroplast repositioning

Before protoplasts divide, chloroplasts cluster around the nucleus and the nucleus centers in the cell (Sheahan et al. 2004a). Both these processes are AF-dependent, and in the case of nuclear centering, also MT-dependent (Katsuta et al. 1990, Sheahan et al. 2004a). Because AFs were required for the establishment and maintenance of vacuolar architecture, we hypothesized that the development of TVS may in turn be required for efficient repositioning of chloroplasts around the nucleus. Indeed, observation of cells in the process of chloroplast repositioning indicated that many cells had chloroplasts transiting through TVS (data not shown). However, an analysis of protoplasts with perinuclearly clustered chloroplasts (after 96 h protoplast culture) showed that only 46% ± 4% (mean with standard error, n = 2) of cells had obvious TVS. Thus, TVS are not strictly required for chloroplast repositioning. Observations of all divided cells, however, indicated that TVS connected perinuclear to cortical cytoplasmic domains and were necessary for centering of the nucleus in the cell, an essential requisite for unbiased chloroplast inheritance (Sheahan et al. 2004a).

Discussion

Vacuole volume, surface area, and architectural complexity increase before cell division

Vacuole volume increased substantially during protoplast culture as cells acclimatized to the hypertonic culture medium. Tonoplast surface area also increased during protoplast culture, with an increasing SA_M-to-SA_C ratio indicating that the formation of IVS contributed significantly to increased tonoplast surface area.

IVS in freshly isolated protoplasts was mainly composed of tonoplast protrusions and small vesicle-like bodies. Protrusions may represent tonoplast infolding in response to osmotic effects of the hypertonic culture medium. Consistent with this hypothesis, Reisen et al. (2005) found that various osmotic stresses caused complex, folded tonoplast morphologies in cultured tobacco cells. Formation of vesicle-like bodies, similar to those observed in this study, have also been shown to occur in response to osmotic stress in cultured tobacco cells (Chang et al. 1996, Reisen et al. 2005). The decrease in the SA_M-to-SA_C ratio during early protoplast culture may therefore represent an adjustment to the hypertonic culture conditions, prior to the onset of ordered vacuolar structuring. It is unclear from this study whether vesicle-like bodies correspond to discrete entities or are part of a continuous system of vacuolar compartments. However, that such bodies often appeared continuous with the peripheral tonoplast suggests the latter option is more likely.

During protoplast culture, simple IVS was replaced by substantive transvacuolar structures, with a threefold increase in the number of cells possessing TVS over the examination period. Mesophyll cells of intact plants lacked complex vacuolar architecture, indicating that the development of TVS in mesophyll protoplasts is not a recovery of the cellular organization present in the progenitor cell type but rather an induction of a new vacuolar architecture. Interestingly, other cell types, particularly epidermal cells, contained numerous TVS. Perhaps this reflects differences in growth characteristics or functional requirements of epidermal and parenchyma cells.

TVS are involved in a number of processes including organelle distribution (Nebenführ et al. 1999), nuclear positioning in dividing cells (Katsuta et al. 1990, Kutsuna and Hasezawa 2002), and delivery of focused defence responses at sites of fungal penetration (Opalski et al. 2005). There exist several potential roles for TVS during protoplast development. In cultured cells that will ultimately divide, the induction of TVS is clearly required for nuclear positioning. It is also probable that TVS enhance the distribution of organelles between cytoplasmic domains, although the ability of chloroplasts to cluster around the nucleus in cells lacking TVS indicates they are not strictly required for this purpose. Indeed, the complex and extensive transvacuolar architecture induced in cultured protoplasts (for example, Fig. 2N) suggests TVS have an additional role.

TVS can commonly be observed in a range of cultured cell types (suspension, protoplast, and callus cultures) from a variety of plant species (for examples, see figures in Traas et al. [1987], Pasternak et al. [2002], Hoffmann and Nebenführ [2004], Rose et al. [2006]). Moreover, cell culture appears to induce TVS formation. Epidermal cells in tobacco leaf explants cultured on basal MS medium (Selga 2003) form TVS, as does callus derived from tobacco leaf explants (M. Sheahan unpubl. results). TVS also form in lily pollen protoplasts cultured in sucrose-supplemented medium (Xu 1992).

Cultured cells are disconnected from the intricate vascular distribution pathways present in the intact plant and in most cases lose or have lost autotrophic capabilities. Continued growth and cell expansion therefore necessitate heterotrophy and the acquisition of nutrients from the surrounding medium. Cell expansion depends on turgor, which in turn requires sufficient vacuolar osmoticum to decrease the effective vacuolar water concentration. Ultimately, this osmotic potential must derive from the transport of ions and metabolites from the culture medium to the cytosol and from the cytosol into the vacuole. Assuming a parallel increase in membrane transporters, the increased tonoplast surface area afforded by formation of extensive transvacuolar architecture would enhance efficient transport between the cytosol and vacuole. In addition, cultured cells must balance the need for metabolites derived from the medium with the need to maintain cytoplasmic homeostasis. Increased tonoplast surface area would help achieve this balance, thus enabling effective heterotrophic metabolism. It is therefore tempting to speculate that the formation of extensive transvacuolar architecture in cultured protoplasts represents an adaptation to heterotrophic cell growth in a hypertonic medium. Interestingly, the vacuolar architecture of cultured cells responds dynamically to stress. For instance, subjecting cultured tobacco cells to sucrose starvation (Rose et al. 2006) or cold stress (Pokorna et al. 2004) dramatically reduces the number of TVS. Collectively these findings indicate that the vacuolar architecture is highly dynamic and responsive to external factors. Further studies are clearly required to define the role of extensive transvacuolar networking.

Vacuole exhibits both actomyosin-dependent and -independent remodeling

In both LatB- and Ory-treated protoplasts, simple IVS, especially vesicle-like bodies, persisted throughout the observation period. This finding suggests that formation of simple vacuolar architecture is independent of the cytoskeleton and perhaps an intrinsic response of the vacuole to hypertonic conditions. Accordingly, the phase of vacuolar remodeling during early protoplast culture (decreased SA_M-to-SA_C ratio) occurred regardless of cytoskeletal disruption. Formation of new, more complex IVS, however, required AFs. MTs also appeared to have a role in IVS formation, as MT disruption slowed the formation of new IVS.

Intriguingly, and contrary to results obtained using tobacco mesophyll protoplasts (Sheahan et al. 2004a), we found here, using GFP-δTIP-expressing *A. thaliana* protoplasts, that both LatB and Ory treatment decreased vacuole expansion. It is possible that over-expression of GFP-δTIP exaggerates the outcomes of cytoskeleton-dependent trafficking of δTIP to the tonoplast. Thus, the effect of LatB and Ory on protoplast and vacuole expansion is visible in δTIP-over-expressing but not wild-type protoplasts. Further studies examining the fluorescence intensity of GFP-δTIP-labeled tonoplast in inhibitor-treated cells may help clarify this issue. Nonetheless, reduced vacuole expansion in LatB- and Ory-treated cells is likely to have contributed to the diminished increase in tonoplast surface area, in turn affecting the ability of treated cells to form IVS.

Consistent with the requirement for AFs in the maintenance of TVS in interphase and dividing cells (Shimmen et al. 1995, Hussey et al. 1998, van Gestel et al. 2002), we found that TVS formation in dedifferentiating protoplasts was also AF- but not MT-dependent. Interestingly, and in contrast to control or Ory-treated cells, where simple IVS converted to substantial IVS and to TVS, the proportion of cells with IVS in LatB-treated cultures remained reasonably static. The differential effect of AF disruption on IVS and TVS is in agreement with two levels of vacuolar structure, one intrinsic to the tonoplast (IVS) and the other AF-dependent (TVS).

Before protoplasts divided, the nucleus positioned centrally within the cell. Both AFs and MTs were required for this process. The primary role of AFs in this process appears to be in formation of TVS. In the fission yeast *Schizosaccharomyces pombe*, nuclear centering is achieved by polymerization of MTs radiating from the nucleus that push against the cell wall (Tolić-Nørrelykke et al. 2005). A similar role for MTs, which relocate from the cell cortex to TVS during the G_2 phase of the cell cycle (Katsuna and Hasezawa 2002), might exist for nuclear centering in plants.

In tobacco BY-2 cells, treatment with BDM leads to a rapid cessation of TVS motility, implying that TVS dynamics are based on myosin-dependent remodeling of actin cytoskeleton organization (Hoffmann and Nebenführ 2004). We examined the role of myosin-based AF dynamics in the formation and maintenance of TVS by treating protoplasts with a number of myosin-disrupting agents. Here, we found that myosin-disrupting agents not only stopped TVS dynamics but also largely prevented the formation of new TVS. Furthermore, adding myosin-disrupting agents to protoplasts with existing TVS significantly decreased the proportion of cells with TVS. These experiments highlight the continuously modulating structure of the vacuole. Because TVS are continually being created and destroyed, the disruption of myosin-based activity permits TVS to be destroyed but not to be recreated. On removal of the inhibitor, therefore, TVS can re-form. Protoplast TVS could be broadly classed as fine and dynamic or large and relatively stable. Fine and dynamic TVS appeared considerably more sensitive to myosin disruption than large and relatively stable TVS. Myosin may thus have two roles in vacuolar structuring, firstly to drive rapid TVS reorganization and secondly to generate forces needed for ramification of TVS through the vacuole. Such dynamic TVS might enhance cytoplasmic mixing and/or solute transport between the cytosol and vacuole, while large and stable TVS might be involved with nuclear positioning.

Our analysis of AFs in myosin-disrupted cells with the GFP-fABD2 fusion protein indicated that transvacuolar AFs were around 10-fold more sensitive to myosin disruption than cortical AFs. While the myosin-disrupting agents had a potentially nonspecific effect on AF polymerization, this finding generally confirms the importance of myosin in TVS dynamics and maintenance. Previous studies have suggested that the bundles of AFs in TVS have uniform directionality (Tominaga et al. 2000). However, recent evidence based on fluorescence recovery after photo bleaching indicates that the highly dynamic TVS present in hypocotyl epidermis of *A. thaliana* may comprise AFs sliding in opposite directions (Sheahan et al. 2004b). It is conceivable that higher-order complexes of myosin dimers, similar to those of myosin II found in muscle sarcomeres, could be involved in TVS dynamics, particularly in the fine, highly dynamic TVS.

The results of these myosin disruption experiments must be interpreted with caution however. BDM and NEM generally have low specificity and can affect a number of cellular processes in addition to myosin function. In animal cells, BDM also inactivates L-type calcium channels (Ferreira et al. 1997) and NEM may potentially alkylate and alter the function of any protein with free sulfhydryl groups. Furthermore, the closest sequence-based homologue of MLCK (the target of ML-7) in *A. thaliana* is annotated in the NCBI GenBank as a putative calcium-dependent protein kinase, unlikely to be related only to myosin function. Nonetheless, the consistent nature of the inhibitory responses of cultured protoplasts to these diverse myosin-disrupting agents argues strongly for a role for myosin motors in the complex phenomenon of TVS architecture and dynamics.

In summary, we show that protoplasts begin with a nearly structureless vacuole but develop a complex vacuolar architecture on reinitiating the cell cycle. Development of a complex vacuolar architecture is actomyosin dependent and contributes to nuclear positioning and possibly an enhanced cellular metabolism, both required for division.

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