Probing cytoplasmic organization and the actin cytoskeleton of plant cells with optical tweezers

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Abstract
In interphase plant cells, the actin cytoskeleton is essential for intracellular transport and organization. To fully understand how the actin cytoskeleton functions as the structural basis for cytoplasmic organization, both molecular and physical aspects of the actin organization have to be considered. In the present review, we discuss literature that gives an insight into how cytoplasmic organization is achieved and in which actin-binding proteins have been identified that play a role in this process. We discuss how physical properties of the actin cytoskeleton in the cytoplasm of live plant cells, such as deformability and elasticity, can be probed by using optical tweezers. This technique allows non-invasive manipulation of cytoplasmic organization. Optical tweezers, integrated in a confocal microscope, can be used to manipulate cytoplasmic organization while studying actin dynamics. By combining this with mutant studies and drug applications, insight can be obtained about how the physical properties of the actin cytoskeleton, and thus the cytoplasmic organization, are influenced by different cellular processes.

Introduction
The actin cytoskeleton is of pivotal importance for many cellular processes, including, among others, cell shape formation, intracellular transport and signalling. It is, as such, a key co-ordinator of the development of eukaryotes. Actin filaments are capable of rapidly forming a plethora of structures, such as cross-linked gels and linear bundles. The organization of actin networks is dependent, besides the intrinsic properties of actin, on a large number of ABPs (actin-binding proteins) [1]. The combined actions of these proteins result in adaptation of the actin organization in response to intra- and extra-cellular cues. In the present review, we focus on an actin configuration that is prominently represented in plant cells: the thick actin filament bundles that play a role in the formation and maintenance of cytoplasmic strands in plant cells. We will discuss how the formation and organization of these actin bundles is regulated and how physical manipulation of cytoplasmic organization with optical tweezers can help to gain insight into these processes.

Cytoplasmic organization in highly vacuolated plant cells
During cell growth, plant cells become highly vacuolated. Mature plant cells contain one or several large vacuoles that can occupy over 90% of the cell volume [2,3]. In intercalary growing plant cells (i.e. cells in which expansion takes place over the whole longitudinal cell axis, resulting in cell elongation), cytoplasm is located around the nucleus (perinuclear cytoplasm) and in the periphery of the cell (cortical cytoplasm). These cytoplasmic areas are interspaced by the vacuole. Strands of cytoplasm bounded by the tonoplast (vacuolar membrane), called cytoplasmic or transvacuolar strands, cross the vacuole to interconnect the perinuclear and cortical cytoplasm (Figure 1). They are formed after cytokinesis [4], during and after cell elongation [5], and during recovery from actin depolymerization [6]. Cytoplasmic strands are highly dynamic: they constantly change shape and location [3,7].

Cytoplasmic strands are thought to function as transport routes for transcripts, proteins and organelles. This transport is visible as cytoplasmic streaming, the myosin-mediated movement of organelles over actin filaments and the hydrodynamic flow induced by this movement [8,9]. All interphase cytoplasmic strands contain actin filaments, and upon actin depolymerization, not only cytoplasmic streaming is inhibited, but also cytoplasmic strands disappear [5,10–16]. Thus F-actin (filamentous actin) not only serves as a transport route, but is also the backbone of cytoplasmic strands. In the present review, we will focus on the structural function of actin filaments in forming, maintaining and reorganizing cytoplasmic strands.

Biogenesis and maintenance of cytoplasmic strands
Two alternative hypotheses have been proposed for the de novo formation of cytoplasmic strands, which are outlined below. (i) Protrusions of cytoplasm extend into the...
vacuolar space and may eventually connect to the peripheral cytoplasm at the other side of the vacuole [15,17]. These protrusions originate from the perinuclear cytoplasm in tobacco BY-2 suspension cultured cells during and just after cytokinesis (Figure 2 and see Supplementary Movie S1 at http://www.biochemsoctrans.org/bst/038/bst0380823add.htm). We propose that these protrusions could be formed by two alternative mechanisms: (a) intact bundles of actin filaments that are positioned against the tonoplast and displaced towards the vacuole by myosin activity indent the tonoplast; or (b) co-ordinated actin polymerization of filaments with their barbed ends towards the tonoplast pushes the tonoplast forward, similar to the protrusion of the plasma membrane of mammalian cells during the formation of lamellipodia or filopodia [17]. (ii) A sheet of the cortical cytoplasm forms an invagination into the vacuolar space, after which the lateral connection between the sheet and the cortical cytoplasm disappears and the sheet becomes a strand, which is connected to cortical or perinuclear cytoplasm at two sides. [18]. Szymanski and Cosgrove [18] present data that show that this type of strand formation indeed occurs in plant cells and propose that the underlying molecular mechanism is myosin activity in the periphery of the cell which pulls a peripheral bundle of actin filaments into the vacuolar space. To decipher how new cytoplasmic strands are formed, the three-dimensional organization of the cytoplasm would need to be studied over time. Owing to the continuous rapid reorganization of the cytoplasmic organization that occurs within seconds, high-speed time series, combined with quantitative data analysis, would be required for conclusive answers.

Once cytoplasmic strands have been formed, they constantly reposition, split and fuse [7]. When myosin activity is inhibited, the cytoplasmic organization is frozen, which indicates that myosin-based displacement of the actin filament bundles that support the cytoplasmic strands is responsible for the reorganization of cytoplasmic strands [7,16]. Experimental application of low concentrations of actin-depolymerizing drugs suggests that actin polymerization does not appear to play a role in the reorganization of existing cytoplasmic strands, other than providing the actin filament bundles that can be displaced by myosin activity [7,16]. Although the number and dynamicity of cytoplasmic strands is known to decrease when cells mature, it is not known how this happens.

**Molecular players in strand formation, maintenance and reorganization**

**Actin-bundling proteins**

Actin filaments in cytoplasmic strands are organized as thick bundles. The actin-bundling protein villin is involved in maintaining these bundles. Injection of an anti-villin antibody in root hairs of Hydrocharis [19] and Arabidopsis [20] causes cytoplasmic strands to fall apart into many thinner cytoplasmic strands, which eventually disappear. This indicates that villin-mediated actin bundling, at least partially, determines the number and the size of cytoplasmic strands. The first plant homologue of villin that was described was isolated from lily pollen [21]. This plant villin bundles F-actin in vitro [22] in a calcium-dependent fashion [23].
**Figure 2** | **Highly dynamic cytoplasmic protrusions into the vacuolar space are formed during and just after cytokinesis**

Three images with 30 s intervals showing the protrusions (black arrows). White arrows indicate the direction of the forming cell plate. N indicate nuclei. Scale bar, 10 μm. Stills are taken from Supplementary Movie S1 at http://www.biochemsoctrans.org/bst/038/bst0380823add.htm.

Although not all plant villins are calcium-dependent [24], the calcium-ion-dependency of some villins indicates that the free cytoplasmic concentration of calcium ions could be involved in controlling the locations and amount of actin filament bundling in plant cells.

Besides villins, several other types of proteins with actin-bundling capacity have been identified in plant cells: fimbrins, formins, LIM domain proteins and EF-1α (elongation factor 1α). The actin cross-linking protein fimbrin is ubiquitously expressed in *Arabidopsis* [25]. The actin binding of the fimbrin AtFIM1 is calcium-independent [26]. Kovar et al. [27] showed that microinjection of Oregon-Green-labelled AtFIM1 in *Tradescantia* stamen hair cells caused the formation of a finer and denser actin filament network in these cells. Microinjection of native AtFIM1 caused inhibition of cytoplasmic streaming and an increased resistance against profilin-induced actin depolymerization [26]. However, when AtFIM1, fused to GFP (green fluorescent protein), was overexpressed in *Arabidopsis*, it appeared to decorate the actin cytoskeleton in different cell types, but did not modify the actin organization [28]. Cellular or developmental defects in fimbrin-knockout lines have not been reported. Thus, although the results from Kovar et al. [26] suggest that fimbrins could play a role in determining the amount of cross-linking between different actin bundles (and thus the number and possibly the number of interconnections of cytoplasmic strands), the results from Wang et al. [28] contradict this. More work is needed to determine whether and how fimbrins contribute to the actin bundles involved in formation and maintenance of cytoplasmic strands.

The actin-nucleating formin AtFH1 is able to bind the side of existing actin filaments *in vitro* so that the newly formed actin filaments form an actin filament bundle together with the existing filament [29]. Michelot et al. [29] suggest that this activity may be the basis of actin bundle formation in plant cells. Whether formin-mediated actin bundling or formin-supported actin bundles are involved in cytoplasmic strand formation remains to be proven.

LIM domain proteins are small (∼200 amino acids) actin-bundling proteins that bind to, stabilize and bundle actin filaments *in vitro* [30,31]. The genome of plants only contains a limited number of LIM domain proteins, in contrast with animal genomes, which contain many [32,33]. Overexpression of the tobacco and lily LIM domain proteins NtWLIM1 and LiLIM1 cause hyper-bundling of actin filaments [30,31,34], indicating that LIM domain proteins, similar to villins, could be involved in forming and/or maintaining actin bundles, and thus be involved in the regulation of the number and size of cytoplasmic strands. LIM-domain-protein-knockouts so far have failed to produce lines which exhibit developmental defects [33].

EF-1α is a protein that functions in binding aminoacyl-tRNA to ribosomes in eukaryotes. In different species, EF-1α binds to microtubules or actin filaments. Gungabison et al. [35] show that maize EF-1α is capable of bundling actin filaments *in vitro* at low pH; this activity is enhanced by the presence of maize ZmADF3 (actin-depolymerizing factor 3).

Although it is likely that besides villins, (some of) these other classes of actin-bundling proteins play a role in the formation and maintenance of actin bundles and thus cytoplasmic strands, disruption of villin activity appears to be sufficient to induce unbundling of actin filaments and disintegration of cytoplasmic strands in root hairs [19,20]. This suggests that either villins are essential for the maintenance of actin bundles that are also supported by other actin-bundling proteins, or that other actin-bundling proteins have functions in processes other than the maintenance of actin bundles in cytoplasmic strands. For example, formins could play a role only during the formation of actin bundles, and not during their maintenance [29].

**Myosins**

The genomes of seed plants contain two distinct groups of myosins, myosin VIII and myosin XI. Myosin VIII localizes to plasmodesmata and the post-cytokinetic cell wall,
endosomes and the ER (endoplasmic reticulum) and thus could be involved in different steps of endocytosis, ER tethering and plasmodesmatal activity. It is unlikely that myosin VIII plays a role in forming or maintaining cytoplasmic strands.

Myosin XI isoforms localize to different organelles such as the ER, mitochondria, plastids, and peroxisomes and peroxisomes. The movement of these myosin XI-decorated organelles over actin filaments is responsible for cytoplasmic streaming. Myosin XI isoforms appear not to be specific for single organelles and analysis of T-DNA (transferred DNA) insertion lines shows that the functions of different myosin XI isoforms are greatly redundant. By application of the myosin ATPase inhibitor BDM (2,3-butanedione monoxime), which freezes cytoplasmic organization, it has been shown that myosins may play a role in the relocation of cytoplasmic strands (see above) [5,7,15,16]. This suggests that the restructuring of the actin bundles that are the backbone of cytoplasmic organization is mainly performed by myosin-based sliding of existing actin filaments, rather than on actin (de-)polymerization. To understand how myosins contribute to the restructuring of the actin filament bundles in cytoplasmic strands, information about the identity, localization, and the activity of these myosins is important to gain an insight into cytoplasmic reorganization.

**Manipulation of cytoplasmic organization**

To fully understand cytoplasmic organization, knowing its molecular aspects is not sufficient. Besides molecular characteristics of the molecules that are involved in the organization of cytoplasm, physical aspects also have to be taken into account. Processes that contribute to cytoplasmic organization not only depend on the molecules involved; they also depend on the physics of these molecules. For example, a single actin filament (30–150 nm) will buckle when pushed against a membrane, whereas a bundle of actin filaments is able to push a membrane forward [52–54]. Also, the degree of cross-linking and bundling dramatically changes the physical properties of an actin network [55,56]. Physical aspects of actin networks can be studied in vitro or within living cells. So far, physical properties of the plant actin cytoskeleton have been probed within living cells only.

Using optical tweezers, physical properties of cytoplasmic organization can be probed in a non-invasive manner. Optical tweezers function by focusing a high-intensity laser beam on a small particle, during which the radiation pressure of the laser constrains the particle to the centre of the laser. Lateral displacement of the focused laser beam allows non-invasive dislocation of these particles [58–61]. Requirements for successful optical trapping are a high numerical aperture lens and the presence of structures in the (sub-)micrometre range, with a higher refractive index than the surrounding medium close to the coverslip. Generally, lasers that emit IR light are used, since these wavelengths do not interfere with imaging and are not perceived by cells. Thus optical tweezers can be employed to produce controlled forces inside living cells to manipulate intracellular organization [62,63].

Optical tweezers have been used to measure the tension in naturally occurring cytoplasmic strands [63,64] and have been employed to alter cytoplasmic organization by the production of new cytoplasmic strands or cytoplasmic protrusions [16]. The tension in naturally occurring cytoplasmic strands was studied by trapping an organelle in one of these strands with the optical tweezers and performing a series of rapid lateral displacements at different laser powers [63]. When performing these experiments in the presence of the actin-filament-depolymerizing drug cytochalasin D (20 μM), tension in cytoplasmic strands was reduced [63], whereas in the presence of the myosin inhibitor BDM (10 mM) the tension in cytoplasmic strands was increased [64]. These experiments show that tension in cytoplasmic strands is produced by actin filaments and suggest that myosin-based sliding of actin filaments is responsible for the deformation of cytoplasmic strands, which fits with the idea that myosin-based sliding of actin filaments is responsible for cytoplasmic restructuring [7,16,18].

Besides measuring tension in existing cytoplasmic strands, optical tweezers can also be employed to modify cytoplasmic organization by trapping an organelle and displacing the trapped organelle into the space occupied by the vacuole. This results in the formation of a cytoplasmic protrusion into the vacuole, bounded by the tonoplast membrane [16]. When the tweezers with a trapped organelle at the top of such a cytoplasmic protrusion were moved to the tonoplast at another side of the vacuole, we have made two different observations: (i) when the organelle is released, for example by switching off the trap, the cytoplasmic protrusion shoots back to its origin and disappears, and (ii) the tonoplast membrane of the protrusion fuses with the tonoplast membrane at the other side of the vacuole, resulting in a cytoplasmic strand that remains intact when the tweezers are switched off (N.C.A. de Ruijter, A.M.C. Emons and T. Ketelaar, unpublished work). The first observation has been seen in tobacco BY-2 suspension-cultured cells; in Tradescantia stamen hair cells, both reactions were observed, and in epidermal peels of onion skin, the second reaction predominantly occurs. Thus the possibility of tweezer-formed strands to fuse with the tonoplast is cell-type- and/or species-dependent.

We have produced tweezer-formed cytoplasmic protrusions in cells in which the actin cytoskeleton had completely been depolymerized (treatment with 500 nM latrunculin B for 12–16 h), or in which the myosin activity had been inhibited (25 mM BDM for 30 min) [16]. It requires more force to produce cytoplasmic protrusions when myosin motor activity is inhibited and less force when the actin cytoskeleton is depolymerized. Thus the actin cytoskeleton increases the deformation capacity of the cytoplasm and myosin inhibition makes the cytoplasm harder to deform. We studied the actin localization during and after physical manipulation of cytoplasmic organization. In control experiments, visible
Cytoplasmic organization: prospects

An integrated optical trapping and confocal microscopy system is a very powerful tool for the investigation of cytoplasmic organization and the underlying behaviour of the actin cytoskeleton. In combination with the use of mutants and drugs to manipulate actin organization it is possible to directly link changes in physical aspects of intracellular organization to the action of specific proteins. Ideally, these experiments should be complemented with experiments using the same techniques in systems with reduced complexity, such as in vitro experiments with purified proteins. Besides these ‘wet’ experiments, the understanding of a complex process such as intracellular organization would probably benefit from an approach in which known aspects of the system are incorporated in models that simplify the complexity, explain the observations and make predictions about unknown aspects of cytoplasmic organization.

References


actin filaments had entered most tweezer-formed strands after several minutes. This suggests that actin filaments occupy free cytoplasmic space, either by active targeting or by random movement. The entry of actin filaments was inhibited by application of BDM [16], but not by partial depolymerization of actin filaments with 100 nM latrunculin B (H. van der Honing, A.M.C. Emons and T. Ketelaar, unpublished work). This suggests that myosin-mediated displacement of existing actin filaments, and not actin polymerization, is responsible for the entry of actin filaments in these strands. Sliding of actin filaments over other actin filaments by myosin activity could be, besides being responsible for reorganization of existing cytoplasmic strands, a mechanism by which actin filaments are pushed against the tonoplast to generate the force to deform the membrane during the formation of new cytoplasmic strands.

Although actin filaments support naturally occurring cytoplasmic strands and appear in tweezer-formed protrusions, the presence of actin filaments does not delay the collapse of cytoplasmic protrusions after tweezer release, indicating that actin filaments do not support tweezer-formed cytoplasmic protrusions, whereas they do support naturally occurring cytoplasmic strands. Apparently, actin filaments can only support a cytoplasmic strand when they are held in place by connections to other actin filaments at both sides of the strand, or else by membrane continuity.

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