Two Distinct Distributions of F-actin are Present in the Hyphal Apex of the Oomycete Achlya bisexualis

Yu Ping Yu 1, Sandra L. Jackson and Ashley Garrill 2

School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8020, New Zealand

We show that two distinct distributions of F-actin are present in the hyphal apex of the oomycete Achlya bisexualis, that have been chemically fixed with a combination of methylglyoxal and formaldehyde and stained with Alexa phalloidin. In approximately one half of the hyphae examined, an F-actin depleted zone within the apical F-actin cap was observed. The remaining hyphae had a continuous apical cap. In live, growing hyphae two types of cytoplasmic organization were observed at the tips, one in which a clear zone was present which may correlate with the F-actin depleted zone, and one where no such clear zone existed which may represent the continuous cap. We suggest that the F-actin depleted zone may be a structural component of the actin network in a subpopulation of oomycete hyphae and may be comparable to similar F-actin depleted zones at the apices of other tip growing cells such as pollen tubes and root hairs. This observation has implications with regard to models of hyphal extension. Hyphae fixed with formaldehyde alone showed continuous apical F-actin caps. Our ability to resolve the F-actin depleted zone likely reflects the cross-linking capabilities of methylglyoxal. The methylglyoxal–formaldehyde combination fixative gave more stained hyphae, brighter staining and more complete staining of F-actin compared to formaldehyde alone.

Keywords: Achlya bisexualis — Actin — F-actin depleted zone — Oomycete — Tip growth.

Introduction

Tip growth is a form of cell extension characteristic of a number of plant and fungal cells that include oomycete and fungal hyphae, pollen tubes, algal rhizoids and root hairs. It is a complex process in which growth is restricted to the apex or tip of the cell, this localization giving rise to tubular cells. Models of tip growth typically espouse the importance of the actin cytoskeleton, which is thought to play a number of roles, including acting as a morphogenic factor (Picton and Steer 1982), acting as a determinant of polarity (Vidali and Hepler 2001), acting as a provider of structural rigidity (Jackson and Heath 1990) and enabling the delivery, filtering and retention of exocytotic vesicles to the growing tip (Miller et al. 1999, Geitmann and Emons 2000).

Investigations of the actin cytoskeleton and its role in tip growth are reliant upon our ability to faithfully observe the structural organization of actin microfilaments. In oomycetes these appear to form an apical cap with a subapical arrangement of fibrils and plaques (Heath 1987). One particular facet that is still poorly understood is a reported F-actin depleted zone that appears to be present in the apex of a number of tip growing cells (Miller et al. 1996, Miller et al. 1999, Roberson 1992). In pollen tubes this has been suggested to be a site where actin is dynamically arranged into microfilaments, the polarized ends of which then impart polarity on the cell (Vidali and Hepler 2001). In oomycete hyphae an F-actin depleted zone has only been reported once (Jackson and Heath 1990) although its existence is a controversial issue as in this study it appeared that not all F-actin was stained. Sub apical regions lacked the brightly stained fibrils and plaques typically seen in fixed hyphae. Jackson and Heath (1990) hypothesized that this may have been due to the low concentrations of rhodamine phalloidin used and/or the blocking of binding sites for the label by actin-binding proteins. It is not known whether these factors led to incomplete staining at the very tip or whether the staining observed indicated the existence of an F-actin depleted zone. This is a critical shortfall in our knowledge as such a structure may well play a critical role in the process of tip growth.

The question of just how closely does what we see resemble the actual situation in a living cell, is the key critique of any study of the cytoskeleton and indeed cell structure. If we are making our observations on live cells then it is generally assumed that we are attaining as close an approximation as possible to the actual distribution of the F-actin. With such cells caution is required in the interpretation of staining patterns as in vivo labeling of actin may stabilize F-actin, may cause over expression artifacts and may block binding sites and thereby compete with actin-binding proteins. Despite these reasons for caution, imaging of live material is the methodology of choice. Due to technical difficulties, however, oomycetes (and indeed most fungi) have proven difficult to load with labels and thus imaging of live cells is, in many cases, not possible. In such circumstances hyphae can be fixed, either chemically or using rapid freeze fixation and observations are made with the

1 Present address: Department of Food and Nutrition, Shih-Chien University, Taipei, Taiwan
2 Corresponding author: E-mail, ashley.garrill@canterbury.ac.nz; Fax, +64-3-364-2590.
assumption that the distribution of F-actin is faithfully preserved by fixation. The validity of this assumption is reliant upon the quality of fixation.

Aldehydes have been widely used as chemical fixatives due to their ability to cross-link proteins (Glauert and Lewis 1998). Of all tested aldehydes, glutaraldehyde is the best cross-linker, as with two functional aldehyde groups, it can rapidly and irreversibly react with a number of amino acids, in particular lysine. This interaction is thought to account for much of its cross-linking (Glauert and Lewis 1998). Its use is problematic, however, when post-fixation labeling involves the use of fluorescent probes such as Alexa phalloidin. Glutaraldehyde-induced fluorescence leads to a high background, which can result in poor contrast. This may mask the fine details of the distribution of F-actin.

An alternative fixative is formaldehyde, which does not auto fluoresce and penetrates tissue more rapidly than glutaraldehyde (Glauert and Lewis 1998). Unfortunately it is not as good a fixative as glutaraldehyde, as with a single functional aldehyde group, it forms fewer and less stable cross-links. Investigators are therefore faced with a situation in which it is possible to get good fixation but poor contrast, or alternatively, sub-optimal fixation but good contrast. The latter alternative is typically preferred with formaldehyde the fixative of choice, although when observations of fine detail are not required the best approach may be a compromise with a fixative comprising low levels of glutaraldehyde in combination with formaldehyde. Regardless of the chosen methodology, the loss of contrast (through the use of glutaraldehyde) and/or structural information (through the use of formaldehyde) may, indeed, mean that our picture of the distribution of F-actin in oomycetes is incomplete.

One aldehyde that has to the best of our knowledge been little used for fixation purposes is methylglyoxal. Like glutaraldehyde it has two functional aldehyde groups, thus it is likely to form many stable cross-links. If it does not cause auto fluorescence, methylglyoxal may give good fixation and enable high-contrast imaging. It is thus important that this is tested as it may impart important information with regard to the actin cytoskeleton and an F-actin depleted zone in oomycetes.

Using hyphae of the oomycete *Achlya bisexualis* we show that fixatives containing a mixture of methylglyoxal and formaldehyde do not cause auto fluorescence and when these are subsequently stained with Alexa phalloidin give higher quality images of the actin network than those fixed with a number of other fixatives. We report the presence in approximately one half of hyphae of F-actin depleted zones in the apical actin cap or an absence of the cap at the very tip. In growing hyphae we report the existence of a cytoplasmic clear zone that may represent the F-actin depleted zone.

Results

All hyphae ($n = 227$) fixed with a combination of 4% formaldehyde and 0.5% methylglyoxal had an apical cap of F-actin that transformed into peripheral plaques and coarse cables in sub apical regions (Fig. 1A–D; see Fig. 3A–C). A subset of these hyphae (48%) had, within the apical cap, a distinct zone that was depleted of F-actin staining (the F-actin depleted zone) (Fig. 1A; see Fig. 3A, B). The remaining 52% showed no evidence of an F-actin depleted zone (Fig. 1C; see Fig. 3C). This zone was not present in hyphae fixed with any of the other fixatives that we tested ($n = 100$ hyphae for each fixative). With

---

**Fig. 1** The effect of various fixatives on the actin-staining pattern and cytoplasm of hyphae labeled with Alexa phalloidin. Fixatives consisted of a combination of 4% formaldehyde and 0.5% methylglyoxal (A, B, C, D), 4% formaldehyde (E, F), 1% methylglyoxal (G, H), 4% glutaraldehyde and 0.5% methylglyoxal (I, J) and 1% glutaraldehyde (K, L). The cytoskeleton in A, C and E is well preserved with an apical cap and subapical plaques. In A an additional structural component, the F-actin depleted zone is evident (arrow). In G there is poor preservation of the cytoskeleton while fine detail is lost in I and K due to poor contrast caused by autofluorescence. DIC images of the cytoplasm (B, D, F, H, J and L) are consistent irrespective of fixative with no evidence of gross cytoplasmic movements. Bar = 5 µm.
Two distinct F-actin patterns in oomycete hyphae

In order to discern whether the appearance of an F-actin depleted zone is due to better preservation of the actin cytoskeleton by the 4% formaldehyde and 0.5% methylglyoxal fixative, relative to the other fixatives, we looked for anything in living growing hyphae that might correlate with such a structure, using DIC optics. When the cytoplasm at the tips of such hyphae was observed, two distinct types of organization were evident. In one of these types there was an apparent clear zone at the very tip where the cytoplasm appeared very smooth (Fig. 2A). The smooth appearance in this zone implies that only organelles and particles that are below the limit of resolution are present. In the other type of organization no such clear zone existed and the cytoplasm had a granulated appearance that was consistent with that seen throughout the hypha (Fig. 2B). Growing hyphae appeared to continually switch between these organizational types, hence the clear zone appeared to be a dynamic structure. We have been unable to observe this clear zone in the cytoplasm of fixed hyphae (see for example Fig. 1B) but its presence may be hidden by the typical reduction in diffraction that occurs with the permeability changes in fixed material.

To further assess the quality of fixation we next examined all fixed hyphae that had been growing at the time of fixation (rather than those that showed the most extensive staining which we have shown in Fig. 1). We restricted this part of the investigation to a comparison of the 4% formaldehyde and 0.5% methylglyoxal and the 4% formaldehyde fixatives as these gave the better actin patterns (as shown in Fig. 1). The combination of methylglyoxal and formaldehyde gave brighter
images and stained more of the actin network than the 4% formaldehyde fixative (Fig. 3A–C compared to Fig. 3D–F). Staining was also more consistent as all hyphae showed comparable staining to those shown in Fig. 3A–C. In contrast, hyphae fixed in formaldehyde gave quite variable results, as shown by the varying degrees of staining in Fig. 3D–F in which we present a well-, a moderately and a poorly stained hypha. We have repeated this experiment on five separate occasions and each time have observed these differences. This result implies that the ability to observe this structure reflects the quality of fixative relative to other aldehyde fixatives.

We further investigated the quality of fixation by monitoring organelle movements as hyphae were fixed. Organelles were selected from various positions relative to the tip and hyphal flank and their movements were observed. A Kruskal-Wallace analysis of variance test showed no significant differences between these mean times taken for all movements to stop after addition of fixative (F >0.05). The mean times (in seconds) (± SD) for complete cessation of movements were 56±36 s for 4% formaldehyde and 0.5% methylglyoxal (n = 6), 111±40 s for 4% formaldehyde (n = 8), 106±132 s for 1% glutaraldehyde (n = 8), 157±80 s for 4% formaldehyde and 0.5% glutaraldehyde (n = 7) and 153±209 s for 1% methylglyoxal (n = 5).

Finally, to determine whether fixation with methylglyoxal causes hyphae to auto-fluoresce we used the combination of 4% formaldehyde and 0.5% methylglyoxal as fixative and compared this to fixatives of 1% glutaraldehyde alone, 4% formaldehyde alone and a combination of 0.5% glutaraldehyde and 4% formaldehyde respectively. Very little auto-fluorescence was observed in hyphae that had been treated with the 4% formaldehyde and 0.5% methylglyoxal fixatives (Fig. 4A) or with formaldehyde alone (Fig. 4B). This contrasted with hyphae that had been treated with fixatives that contained glutaraldehyde. In these hyphae background fluorescence was observed (Fig. 4C, D).

**Discussion**

Previous studies of the actin distribution in formaldehyde-fixed oomycete hyphae have shown a cap of actin filaments, adjacent to the apical membrane, and a sub-apical network of widely spaced fibrils and plaques (Heath 1987). In this study we show that the incorporation of methylglyoxal in the fixative gives a similar overall actin pattern, but in approximately one half of hyphae, an additional structural component, the F-actin depleted zone is visible. This has previously only been described in live, electroporated hyphae (Jackson and Heath 1990), although there are indications that in that study not all actin may have labeled (see below). Irrespective of this, to the best of our knowledge, this paper represents the first report of an F-actin depleted zone in chemically fixed oomycete hyphae. We suggest, for the reasons given below, that the ability to observe this structure reflects the quality of fixative relative to other aldehyde fixatives.

While an actual quantification of the quality of fixation is difficult, in comparing actin staining patterns of hyphae that were fixed with the combination versus methylglyoxal or formaldehyde alone, we were able to observe the overall F-actin distribution typical of oomycetes more frequently. This suggests that across a sample of hyphae the fixation and subsequent staining is much more consistent with the formaldehyde–methylglyoxal combination. Secondly, with the combined fixative the fluorescence of labeled actin filaments was brighter. This suggests that a higher number of actin filaments remained and were accessible in these cells, following fixation. Thirdly, all of the cells had a more extensively labelled network whereas only 38% had comparable extensive labeling with formaldehyde alone. The remaining 62% of cells had regions of incomplete staining providing further evidence that the cross-linking of the filamentous actin network was better with the combination fixative. Finally, it is worth noting analogous work on pollen tubes. It was initially thought from conventionally fixed cells that there was a dense concentration of actin in the tips (Pierson 1988). More recent work, however, using techniques that are likely to preserve cellular structure better, such as rapid freeze fixation and green fluorescent protein in live cells, suggests that there is limited detectable F-actin in the tips.
(Vidali and Hepler 2001, Geitmann and Emons 2000). Whether these F-actin depleted zones are completely free of F-actin is open to debate and they may represent areas of delicate, unstable F-actin, such as those described in pollen tubes (Gibbon et al. 1999) and also in the tips of algal rhizoids (Hable et al. 2003).

The improvement in fixation may be due to structural similarities between methylglyoxal and glutaraldehyde. Both are dialdehydes that primarily cross-link lysine amino acid residues. Glutaraldehyde is thought to be such a good fixative because its two functional aldehyde groups can react rapidly and irreversibly with proteins (Glauert and Lewis 1998). Methylglyoxal is also a dialdehyde and should offer comparable cross-linking to glutaraldehyde without, as our data demonstrates, the problems of auto fluorescence. We suggest that the use of a combination of 0.5% methylglyoxal and 4% formaldehyde may preserve the cytoskeleton of oomycetes better than formaldehyde alone. While not regarded as the best cross-linker, formaldehyde has been widely used as a fixative and, on its own, has revealed an F-actin depleted zone in eufungal hyphae (Roberson 1992). It penetrates tissue rapidly which may be of importance, given the recent suggestion of Foissner et al. (2002) that an organelle-free zone in pollen tubes was better preserved by fast fixation rather than the gradual introduction of fixatives. We suggest that our combination fixative offers fast penetration (formaldehyde) and subsequent irreversible cross-linking (methylglyoxal), without the problems of auto fluorescence that are evident with glutaraldehyde.

Despite an improved methodology for chemically fixing hyphae, it is still unclear as to how closely the actin distributions that we have observed match the actual actin distributions in a living, growing hypha. It should be remembered that our observations have been made on fixed hyphae and therefore do not address the spatial and temporal dynamics of the actin as a hypha extends. A definitive study requires the imaging of all actin at high resolution and sensitivity in a living cell (Heath 2000). This is theoretically possible with the use of GFP-actin or GFP-talin, but in hyphal organisms this approach has yielded limited success (Doyle and Botstein 1996). GFP-talin has been used with tip-growing pollen tubes and interestingly an F-actin depleted zone in the tips was observed (Kost et al. 1998). Alternatively, an actin label can be incorporated into live cells by electroporation or microinjection and these techniques have, like GFP-talin, revealed F-actin depleted zones in pollen tubes (Miller et al. 1996, Anderhag et al. 2000). Unfortunately, reports using electroporation or microinjection on hyphal organisms are limited, presumably due to technical difficulties.

In one of the few such reports, an F-actin depleted zone similar to the zone observed in this work was reported, although, in contrast to the current and to other studies, the sub apical peripheral actin network of plaques and cables was not present suggesting that not all F-actin may have labeled with rhodamine phalloidin (Jackson and Heath 1990). It is possible that the electroporation technique or the probe affected the F-actin localization and/or labeling and this may account for the F-actin depleted zone at the tip.

It is tempting to speculate that the clear zone that we observed in the tips of growing hyphae represents the site of the F-actin deplete zone and that it may be analogous to the apical clear zone described in pollen tubes (Lancelle and Hepler 1992). The appearance and disappearance of this clear zone in hyphae is unlikely to be caused by the movement of vacuoles toward and then away from the tip as vacuolar movements of this type typically occur when growth stops and then restarts. It is possible that the lack of a diffraction pattern in this area may be indicative of a lack of F-actin thereby removing any actin-based particle movement. In pollen tubes the clear zone represents an area that is devoid of larger organelles such as mitochondria and Golgi and it has been hypothesized that these are either filtered out by sub-apical fine F-actin (Kost et al. 1998) or undergo stratification on the basis of their size (Vidali and Hepler 2001). Similar organelle distributions have been described in freeze-substituted hyphae of the oomycete Saprolegnia ferax, with larger organelles such as mitochondria, Golgi and nuclei largely excluded from the most apical 5 μm, an area that is abundant in wall vesicles (Heath and Kaminskyj 1989).

Perhaps one of the more intriguing questions that this study raises is how an F-actin depleted zone might fit into models of hyphal tip growth. Contrasting models suggest that actin may either resist force at the tip, if growth is powered by turgor pressure, or may provide a protrusive force at the tip, if growth is akin to amoeboid movement in animal cells (Heath 1995). Such protrusive forces in animal cells are thought to arise from the insertional polymerization of actin microfilaments (Pantaloni et al. 2001). There have been suggestions that both turgor-driven and amoeboid models may be correct and that, depending upon environmental conditions, both types of growth may occur concurrently in different hyphae that constitute a single mycelium (Garrill 2000). In both turgor-driven and amoeboid growth models cell wall precursor containing vesicles would need to be transported to and be exocytosed at the tip. The F-actin depleted zone may present a means to allow the internal determination (and hence regulation) of an area that would more readily yield to turgor pressure or, alternatively, a protrusive force. It may also enable the exclusion of larger organelles at the tip and would also presumably represent the major site of exocytosis of wall material. With this in mind it is interesting to note the finding of Ketelaar et al. (2003) that a localized instability of the actin network in root hairs can determine the site of exocytosis.

In summary, we present an improved methodology for the chemical fixation of oomycete hyphae that reveals an F-actin depleted zone at the tips of oomycete hyphae. The significance and universality of this F-actin depleted zone will be a critical debate for biologists to resolve in the future if we are to understand the mechanism of tip growth.
Materials and Methods

Hyphae of the oomycete A. bisexualis Coker (available from the University of Canterbury culture collection) were grown on cellophane strips overlaying PYG media (Lundy et al. 2001). Prior to fixation hyphae were cut 1 cm behind the growing tip and immobilized in a thin layer of 2% low melting point agarose (Sigma). They were covered with liquid PYG and left for 30 min to allow growth to resume.

For fixation the relevant aldehyde(s) was/were made up (at the relevant concentrations) in 50 mM PIPES (Sigma, St. Louis, MO, U.S.A.) buffer (adjusted to pH 6.8 with KOH (BDH)). Various concentrations (50, 60, 80, and 100 mM respectively) of buffer were tested in preliminary experiments to determine the optimal concentration. Fifty mM was found to give the best preservation and was thus used for all subsequent experiments. Hyphae were fixed with a mixture of 0.5% methylglyoxal (Sigma) and 4% formaldehyde (ProSciTech., Thirringgowa Central, Queensland, Australia), 4% formaldehyde, 0.5% glutaraldehyde and 4% formaldehyde, 1% methylglyoxal or 1% glutaraldehyde (Ted Pella Inc, Redding, CA, U.S.A.) for 30 min and then rinsed twice in buffer solution.

Staining of the actin cytoskeleton was accomplished using Alexa Phallolidin ( Molecular Probes) according to the method of Heath (1987). The sample was covered with tin foil (to prevent photo bleaching) for 30 min and then washed twice with buffer solution, each time for 15 min. The sample was covered with tin foil between washes. After removal of the second wash the antifading agent p-phenylenediamine (Sigma) was added (made up to 0.1% (w/v) in dd H2O). Samples were examined immediately after staining using an MRC1024 confocal microscope (Bio-Rad, Mississauga, Ontario, Canada) or an Olympus BH-2 Epifluorescent microscope.

We restricted our observations to those hyphae whose morphology and F-actin staining pattern indicated that they had been growing at the time of fixation. This approach is necessary as the structure of the F-actin network differs between growing and non-growing hyphae. Hyphae that had rounded rather than tapered tips and those that did not stain were excluded from the analysis.

Acknowledgments

We thank Juliet Gerrard and Antonia Miller for the gift of methylglyoxal and Craig Galilee and Matthew Walters for technical assistance. This research was partially funded by a University of Canterbury Doctoral Scholarship. Yu was supported by a University of Canterbury Doctoral Scholarship. This research was partially funded by a University of Canterbury Doctoral Scholarship.

References


(Received June 24, 2003; Accepted December 15, 2003)