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Spitzenkörper, vacuoles, ring-like structures, and mitochondria of *Phanerochaete velutina* hyphal tips visualized with carboxy-DFFDA, CMAC and $DiOC_6(3)$

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ABSTRACT

Growth and organelle morphology in the wood rotting basidiomycete fungus *Phanerochaete velutina* were examined in Petri dishes, on agar-coated slides, and in submerged cultures, using DIC, fluorescence and four-dimensional (4-D; x,y,z,t) confocal microscopy, with several fluorescent probes. *Phanerochaete* is ideal for this work because of its fast growth, robustness, and use in a wide range of other studies. The probe carboxy-DFFDA, widely used for labelling vacuoles, has no effect either on hyphal tip extension or colony growth at the concentrations usually applied in labelling experiments. Carboxy-DFFDA labels the vacuoles and these form a tubular reticulum in hyphal tip cells. The probe also labels extremely small vesicles (punctate fluorescence) in the apex of tip cells, the Spitzenkörper, and short tubules that undergo sequences of characteristic movements and transformations to produce various morphologies, including ring-like structures. Their location and behaviour suggest that they are a distinct group of structures, possibly a subset of vacuoles, but as yet to be fully identified. Regular incursions of tubules extending from these structures and from the vacuolar reticulum into the apical dome indicate the potential for delivery of material to the apex *via* tubules as well as vesicles. Such structures are potential candidates for delivering chitin synthases to the apex. Spitzenkörper behaviour has been followed as hyphal tips with linear growth encounter obstacle hyphae and, as the hydrolysis product of carboxy-DFFDA only accumulates in membrane-enclosed compartments, it can be inferred that the labelled structures represent the Spitzenkörper vesicle cloud. Mitochondria also form a reticular continuum of branched tubules in growing hyphal tips, and dual localisation with $DiOC_6(3)$ and CMAC allows this to be distinguished from the vacuolar reticulum. Like vacuolar tubules, mitochondrial tubules also span the septa, indicating that they may also be a conduit for intercellular transport.

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Introduction

The tips of fungal hyphae contain polarised organelle systems that keep pace with the extending apex. Instrumental in controlling polarised growth is the Spitzenkörper at the extreme hyphal tip. Its presence is required for growth and its position predicts the direction of growth (Bartnicki-García *et al.* 1995; Sudbery & Court 2007). Polarised organelle systems include the vacuoles, which can be tubular and motile and may form a reticulum along hyphal tip cells in a range of filamentous fungi (Rees *et al.* 1994; Ashford & Allaway 2007; Watkinson *et al.* 2005). Although there have been significant advances in understanding organelle targeting and fusion at the molecular level (see Ashford & Allaway 2007 for references), the activity and interactions of organelles at a higher level of organisation are much less well understood. Mitochondria are also known to form networks in many cell types but have been little studied in hyphal tips. There are now powerful tools to study organelle dynamics and other cellular events in living cells, using fluorescence or laser scanning confocal and multiphoton microscopy after labelling with specific fluorescent probes (see references in Ashford & Allaway 2007 and Bourett *et al.* 2007). In the present study hyphal tips of *Phanerochaete velutina* were labelled with three fluorescent probes: carboxy-DFFDA and CMAC for vacuoles and $DiOC_6(3)$ for mitochondria, and examined using fluorescence and four-dimensional (4-D) confocal microscopy.

In live imaging it is important that hyphae are healthy and have not been modified by the procedure. Cutting and lifting out colony segments and placing them on a new substrate has known deleterious effects on hyphal tips (Allaway *et al.* 1997). Even in mildly stressed hyphae, growth rates and cell shape may be altered leaving permanent changes in hyphal tip morphology (Bartnicki-García *et al.* 1995). It is generally assumed that fluorescent probes at the low concentrations usually applied have little to no toxic effect, but this is rarely tested. Hyphae of many fungi do not extend quickly enough to test direct effects of probes on hyphal tip extension rates, and damage is usually monitored by changes in the appearance of cytoplasm and organelles in the treated cells, which is not ideal. Using Petri dish culture we determined whether carboxy-DFFDA at the concentration used to label organelles has an effect on growth. We also examined growth and organelle distribution in agar-slide culture, where disturbance is minimal, and compared results with the culture system commonly used for fluorescence and confocal microscopy, where hyphal wedges are cut from the growing edge of Petri dish cultures and examined submerged in the test solution, in closed chambers. *Phanerochaete velutina*, a fast-growing, cord-forming saprotroph belonging to the *Basidiomycota*, is an ideal fungus to use. It has an extensive tubular vacuole system, which is known to be a conduit for nutrient translocation (Watkinson *et al.* 2005; Darrah *et al.* 2006; Bebber *et al.* 2007) and its rapid growth over short time periods, and branching in culture, make it ideal for studying organelle dynamics in relation to growth, morphogenesis, and transient changes in the environment.

In addition to the expected labelling of the vacuolar reticulum, carboxy-DFFDA also labelled other structures in actively growing hyphal tips, notably the Spitzenkörper, very small vesicles and short tubules that formed characteristic ring-like structures. We investigated these further using 4-D (*x*,*y*,*z*,*t*) confocal microscopy of continuously loaded hyphal tips. The observations show that carboxy-DFFDA is an excellent alternative to FM4-64 for labelling the Spitzenkörper, enabling its behaviour to be studied in conjunction with that of other organelles in the apex, such as the tubular vacuole system. Furthermore, it labels intact compartments by accumulating in them, rather than only labelling membranes. Mitochondria were labelled with $DiOC_6(3)$, and they also formed networks, which spanned the septa. The examination of tubules that transform into ring-like structures in growing hyphal tips by 4-D confocal microscopy rather than as a single optical section provides an opportunity to evaluate their morphological transformations and dynamic behaviour, emphasizing their distinct appearance and activity, and suggesting that they may be distinct from the vacuolar system.

Materials and methods

Cultures and reagents

Cultures of *Phanerochaete velutina* (DC. ex Pers.) P. Karsten from Lynne Boddy, University of Cardiff, were maintained on 2 % malt extract agar [2 % (w/v) Oxoid malt extract plus 2 % (w/v) Oxoid No. 3 agar (Oxoid Limited, Basingstoke, Herts, UK)] at 22 ± 1 °C in the dark.

Fluorochromes, from Molecular Probes (Eugene, OR), were as follows: Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA, O-6151, stock solution 10 mg ml^{-1} in DMSO or, for confocal microscopy, in acetone), 7-amino-4-chloromethylcoumarin (CMAC, C-2110, stock solution 5 mg ml^{-1} in DMSO), and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, D-273, stock solution 10 mg ml^{-1} in MeOH). All stock solutions were stored at -20 °C and diluted with reverse osmosis (RO) water to the appropriate concentration just before each experiment.

Preparation of the slide cultures

Sterilised glass microscope slides (76.2 mm × 25.4 mm, 1–1.2 mm thickness) were coated with autoclaved (15 p.s.i. for 15 min) 1 % agar containing 2 % malt, by dipping them in a beaker of warm (*ca* 60 °C) liquid medium for about 5 s. They were briefly drained by touching the side of the beaker and laid horizontally on a bent glass rod, placed on a moist filter paper disc in a standard (10 cm) glass Petri dish. All operations were carried out under sterile conditions. The slide was inoculated at its centre with a plug (*ca* 7 mm diam) of hyphae cut just behind the margin of a one-week-old culture grown on agar in a Petri dish, using a cork borer. The inoculated slide culture was incubated at 23 °C in the dark. A slide culture with a 4-d-old colony is shown in Fig 1.

Microscopy and imaging

Micrographs were taken on a Zeiss Axiophot microscope with DIC or epifluorescence optics. Filter combination BP450-490, FT510, and LP515-585 was used for fluorescence with

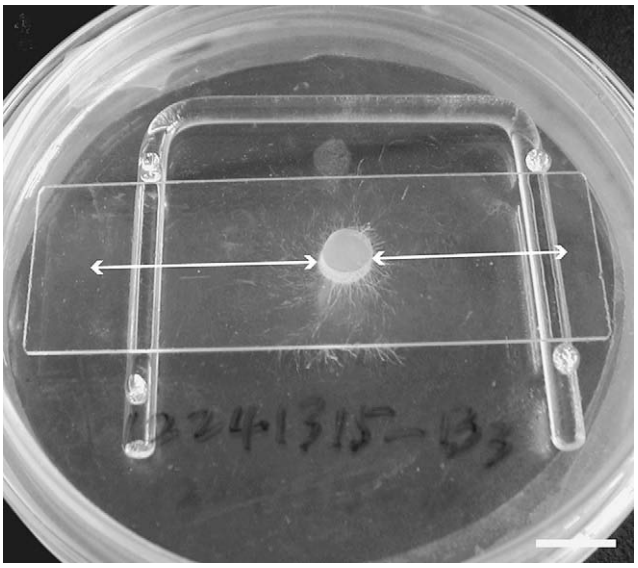

Fig 1 – Slide culture system. An agar-coated microscope slide is placed on a bent glass rod (diameter 4 mm) resting on moist Whatman No. 1 filter paper in a Petri dish sealed with a strip of Parafilm™. Axes for measurement of colony growth are indicated by arrowed lines. Bar = 10 μm.

carboxy-DFFDA and $DiOC_6(3)$; and BP365, FT395 and LP420 for CMAC. Individual images or sequences of images were captured with an Image Point CCD camera (Photometrics, Tucson, AZ) connected to a Dell Precision 420 PC computer using AxioVision 3.1 software (Carl Zeiss, Germany). Confocal microscopy of hyphal tips was undertaken at Department of Plant Sciences, University of Oxford, and all procedures were as described in Darrah *et al.* (2006), except that hyphae illustrated here were continuously loaded from a solution containing 5 μM carboxy-DFFDA throughout the observation period.

Comparison of mycelial growth in slide and Petri dish cultures

Growth in the slide culture system was compared with that on the same agar medium in standard Petri dishes. To measure colony growth, the distance between the leading edge of the mycelium and the edge of the inoculum plug was measured at daily intervals under a dissecting microscope. Measurements were made along two equidistant radii in each Petri dish and then averaged ($n = 5$ plates per treatment). Colony growth in the slide culture system was measured in the same way along the two radii parallel with the long axis of the slide ($n = 5$) as indicated in Fig 1. Data were analysed throughout by Student's *t* test.

Effect of carboxy-DFFDA on mycelial growth in slide cultures

To determine the effect of carboxy-DFFDA on colony growth, 2-d-old slide cultures were treated in each of three ways: (1) a solution of 500 μl carboxy-DFFDA was applied to the colony for 5 min and rinsed off with the same volume of RO water for 1 min; (2) the sample was treated with 500 μl carboxy-DFFDA for 5 min and then rinsed with the same volume of carboxy-DFFDA for 1 min; or (3) the sample was treated with 500 μl RO water for 5 min and then rinsed with the same volume of RO water for 1 min (control). Untreated colonies of the same age were also used as controls. There were five replicates for each treatment. Measurements of colony growth were made at daily intervals as described above.

Effect of carboxy-DFFDA on extension of individual hyphal tips

After 2.5–3 d of colony growth at 23 °C in slide culture the inoculum plug was carefully cut around its edge and removed from the slide. A solution of 500 μl of 0.5 μM carboxy-DFFDA in RO water was then gently dropped on to the agar surface just behind the hyphal tips and allowed to spread to submerge the mycelium totally. The lid was replaced and slides were incubated for 5 min in the dark after which cultures were rinsed with RO water for 1 min. Two Teflon spacer strips (12 mm PTFE Tape, Klingerflon, Klinger GmbH, Idstein, Germany) were placed on the slide beyond the edge of the colony and a No. 1 cover slip was applied. Controls were treated in exactly the same way but with RO water instead of carboxy-DFFDA solution. Tip extension was recorded for individual hyphae by consecutive image capture digitally under DIC optics at 10 min intervals for sustained periods of time. Hyphae with regular and irregular hyphal morphologies were distinguished in the measurements. The slide remained undisturbed on the microscope stage at approximately 23 °C throughout. The colony was kept moist by the addition of 100 μl of RO water at intervals of 30 min. Only one hypha per slide was measured and this was irradiated only during image collection. Individual hyphal tips were followed for periods of up to 5 h.

Visualization of organelles in the slide culture system

Hyphal tips were prepared as described for measurements of individual hyphal tip extension. Hyphae were labelled with 0.5 μM carboxy-DFFDA for 5 min with or without a 1 min rinse in RO water for visualisation of the vacuole system. Mitochondria were labelled with 0.55 μM $DiOC_6(3)$ for 10 min followed by 1 min rinse in RO water. The vacuole system and mitochondria were visualised simultaneously by dual labelling with 14.4 μM CMAC for 15 min, followed by 0.55 μM $DiOC_6(3)$ for 10 min, with a 5 min rinse prior to observation.

Results

Radial colony growth in slide and Petri dish cultures and the effects of carboxy-DFFDA

Colony growth rate in slide and Petri dish cultures increased over the experimental period such that the final rate was significantly different from the rate over the first day ($P < 0.05$, Fig 2A). Growth in slide and Petri dish cultures did not differ significantly at any point in time over the 4 d growth period ($P > 0.05$). When solutions of carboxy-DFFDA or RO water were applied in various combinations to the slide cultures and compared with the control where no solution was added (Fig 2B), it was found there was no significant effect of either

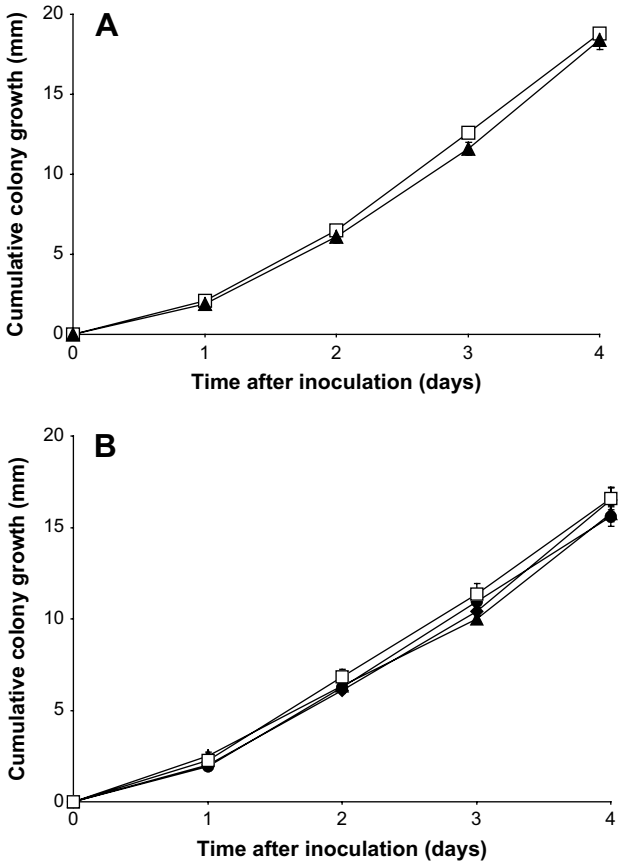

Fig 2 – (A) Comparison of colony growth in Petri dish (—□—) and slide (—▲—) culture. Means ± standard errors (S.E.M.) are shown ($n = 5$): where the standard error is very small it is concealed by the symbol. (B) Effect of carboxy-DFFDA on cumulative colony growth in slide culture. Colony treated for 5 min followed by a 1 min rinse. Untreated control (—□—), treated and rinsed with RO water (—▲—), treated with carboxy-DFFDA and rinsed with RO water (—◆—), treated and rinsed with carboxy-DFFDA (—●—). Means ± S.E.M. are shown ($n = 5$).

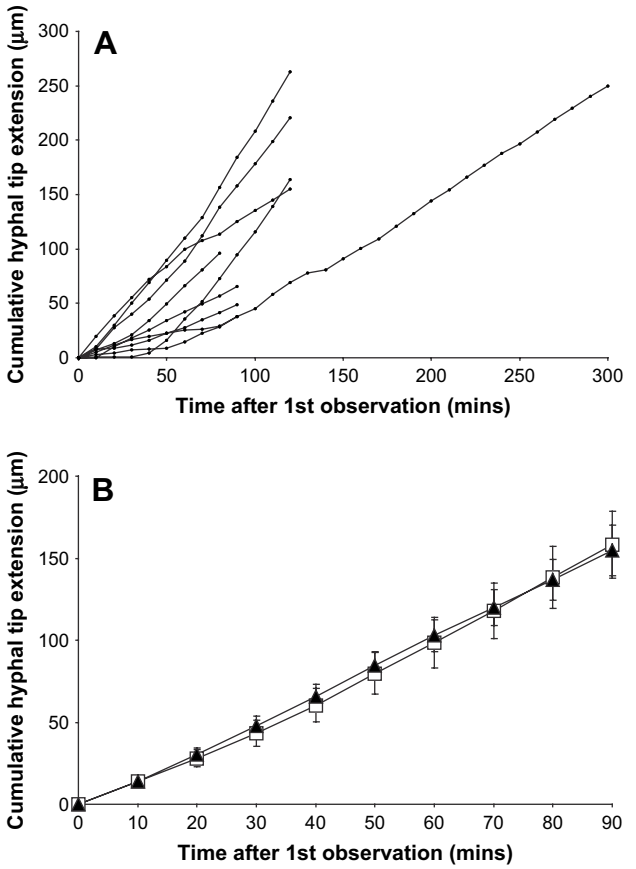

Fig 3 – (A) Extension of individual hyphal tips in slide culture measured under DIC optics at 10 min intervals. Each line represents consecutive values for one hyphal tip; only one hypha per slide was measured. Measurements were made on 3-d-old colonies. (B) Effect of carboxy-DFFDA on hyphal tip extension in slide culture. Colony treated with RO water (—□—) or carboxy-DFFDA (—▲—) for 5 min and rinsed with RO water for 1 min. Means ± S.E.M. are shown ($n = 5$).

carboxy-DFFDA or the procedure to add solution on colony growth. Treatments were not significantly different from each other ($P > 0.05$), and no treatment was significantly different from the control at any stage in the experiment, but the final growth rate was significantly different from the initial rate in all treatments and the control.

Extension of Phanerochaete *hyphal tips under DIC optics*

The mean length of *Phanerochaete velutina* tip cells in the slide culture system was 537 ± 22 μm ($n = 30$) and the mean diameter was 10 ± 0.5 μm ($n = 30$). Individual hyphal tips extended at very different rates (Fig 3A) but, provided that the slide cultures were regularly replenished with RO water during microscopical observation, hyphal tips continued to extend at sustained rates for at least 4–5 h in both control and carboxy-DFFDA treated mycelia. Some hyphae showed an initial period of slow extension for up to 50 min before a sustained extension rate was achieved (Fig 3A). They did not appear to be deleteriously affected by the irradiation necessary to record the DIC image. Carboxy-DFFDA had no significant effect on hyphal tip extension ($P > 0.05$, Fig 3B).

Various patterns of hyphal tip morphology were observed and their extension monitored under DIC optics. Fig 4A–E shows extension of one hyphal tip of typical morphology in the slide culture, in this case treated with carboxy-DFFDA (average extension rate 1.55 ± 0.02 μm min^{-1}). The Spitzenkörper was usually obvious under DIC optics in rapidly extending hyphae and is seen in Fig 4A–E. Fig 4F, G shows an advancing tip of undulating appearance, caused by irregular extension (average extension rate 1.52 ± 0.19 μm min^{-1}). The average extension rate of tips of this morphological type was significantly lower than that of tips of more regular typical morphology (Table 1). These different morphologies were found in both control (treated with RO water) and carboxy-DFFDA treated hyphae and extension rates of the fast, regular, and slower irregular hyphal types were not significantly different between the two treatments. It was very common to find tips that had become constricted at one point and

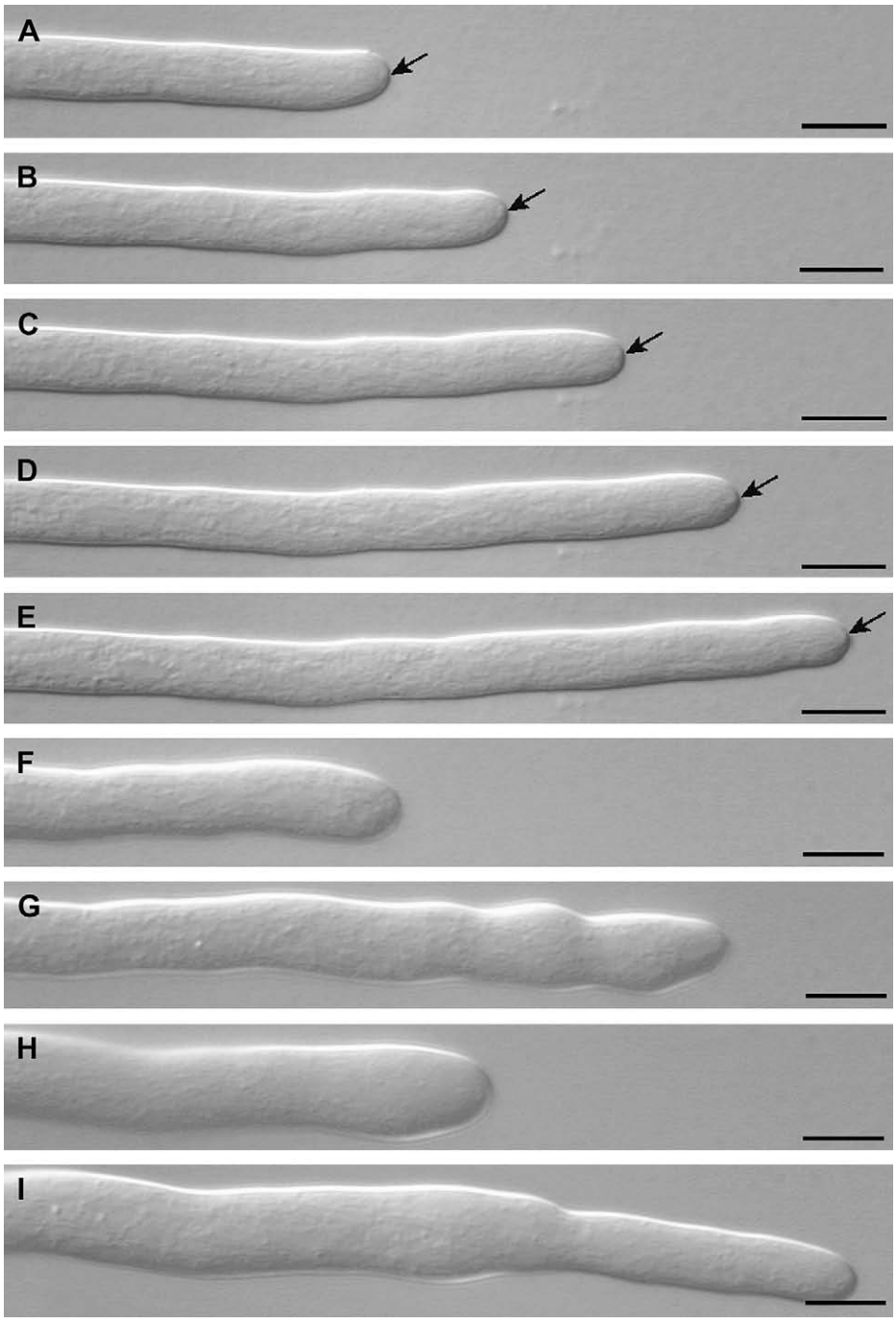

Fig 4 – Range of form of growing apices. (A–E) Extension of a hyphal tip of typical appearance (extension rate 1.55 ± 0.02 μm min^{-1}). Note the Spitzenkörper (arrow). (F–G) Hyphal tip of irregular appearance (extension rate = 1.52 ± 0.19 μm min^{-1}). (H–I) Extension of a narrowed apex (extension rate = 1.67 ± 0.33 μm min^{-1}). Extension rate measured at 10 min intervals. Bars = 10 μm.

continued to extend as a narrower hypha. Fig 4H, I shows the typical extension of a narrowed tip in a rapidly extending hypha (1.67 ± 0.33 μm min^{-1}). The narrowed region sometimes became the site of a branch point. Hyphal tips also branched dichotomously (Fig 5A–G). These tips usually showed little or no extension and a change in the shape of the apex before branching became obvious (Fig 5A–C). Hyphae also showed reduced extension rates or even stopped extending at low moisture levels or following disturbance from manipulation.

Labelling of vacuoles, mitochondria, and Spitzenkörper

The vacuole system in the tip cells of *Phanerochaete* hyphae, labelled with carboxy-DFFDA and viewed by epifluorescence

Table 1 – Comparison of average extension rates of regular and irregular hyphal tips with and without carboxy-DFFDA over 10 min intervals

Hyphal morphology	Hyphal extension per minute					
	carboxy-DFFDA			RO water		
	Mean ± S.E.M. (μm)	Range (μm)	N	Mean ± S.E.M. (μm)	Range (μm)	N
Regular	2.04 ± 0.08 [a]	0.57–4.40	9(61)	2.07 ± 0.09 [a]	0.78–3.44	9(54)
Irregular	1.13 ± 0.14 [b]	0.34–2.53	9(29)	1.17 ± 0.09 [b]	0.001–3.26	8(35)

N = number of hyphae (= number of measurements). Rates with different superscripts are significantly different at $P < 0.05$.

microscopy *in situ* on the culture slides, showed typical range of form, from a reticulum (Fig 6A) to clusters of small, less motile vacuoles (Fig 6B). In addition, in many labelled hyphae the tip had a 'cloudy' appearance due to fluorescence and there was also a distinct patch of more intense fluorescence just beneath the apex at the predicted location of the Spitzenkörper (Fig 6C).

With $DiOC_6(3)$ mitochondria became labelled and often also formed a tubular reticulate system. This elongate mitochondrial system was readily differentiated from the tubular vacuole system in dual-labelling experiments where vacuoles were labelled with CMAC (Fig 7A). Tubular systems could also be seen using DIC optics, but could not be differentiated from each other (Fig 7B). Mitochondria ranged in form from spherical to elongate, and in length from 0.5–20 μm, with a diameter of 0.5–1 μm. They mostly appeared to be parallel with the long axis of the cell but could cross the cell at an angle. Labelled mitochondria were often concentrated near the apex, but otherwise appeared fairly evenly distributed throughout the rest of the tip and penultimate cells. In cells more distant from the apex mitochondria became fewer and were mainly concentrated at the cell periphery. The mitochondria were motile: they extended or contracted, separated, and fused with one another. Their movements appeared to be directional and non-random. Both vacuolar and mitochondrial tubule tips underwent a series of searching/contacting movements with other tubules of their respective systems. Sometimes these were followed by fusion and in other cases not. Mitochondria were also seen to cross apparently mature septa between the tip and penultimate cells (Fig 7C, D). The DIC image illustrates the septum and its clamp as well as the vacuole system which was not in this case labelled with a probe but can be seen to consist of spherical vacuoles (Fig 7D).

To improve resolution and gain more information, extending tips of hyphae, continuously loaded with carboxy-DFFDA, were examined by confocal microscopy (Figs 8–10). These were usually still extending vigorously when experiments were terminated at >1 h after the first observation under the microscope. They gave similar results to hyphae loaded for 10 min and rinsed before observation and did not appear to sustain damage, but staining was usually more intense. This applied to all structures that accumulated the hydrolysis product of the probe (carboxy-DFF), i.e. tubular vacuoles, short paired tubules that transform into forked and/or ring-like structures, and an apical patch in the region of the Spitzenkörper. In addition a region of varying length at the anterior end of most tip cells appeared cloudy; this fluorescence was minutely punctate, not diffuse.

Vacuoles rapidly over-stained in these continuously loaded samples. However characteristic vacuole forms were present and the vacuole system showed the usual polarity and kept pace with the advancing hyphal apex, as it extended. Rapidly extending tips were usually dominated by a mostly tubular vacuole system connected into a reticulum with a few small spherical vacuoles, while slowly or non-extending tips contained mostly spherical less motile vacuoles. Spherical vacuoles that were less motile increased in frequency and size with distance from the tip. Penultimate and more basal cells tended to contain larger less motile, though still interconnected, vacuoles. In contrast, just prior to branching, in regions of the penultimate cell where a branch would develop, the vacuole system reverted to a tubular reticulum and the reticulum extended into the new branch.

The position and activity of the apical patch of fluorescence indicates that a component of the Spitzenkörper had become labelled. It was usually present in extending hyphae and was not seen in non-extending apices. In a typical hyphal tip undergoing linear extension (1.80 μm min^{-1}) the fluorescent patch was continuously present in an anterior position at the centre of the apical dome (Figs 8, 10). It kept pace with the advancing tip to maintain a more or less constant distance from the apical pole. It was not stationary at any stage, but was seen to undergo very small movements laterally to left and right of centre, while still maintaining its overall position at the leading edge of the hyphal tip (Fig 8: left of centre 64–96 s, 192–208 s; central 16–48 s, 144–160 s; right of centre 128 s; Supplementary Material). It also underwent non-random minor changes in shape, appearing as if fluorescent vesicles were being alternately added and removed from its edges and from time to time appeared as a ring of fluorescence around a dark core region (e.g. Fig 8 at 48 s, Fig 9 at 544 s, Fig 10 at 1008–1024 s) and the overall intensity of its fluorescence also varied (Fig 8). A number of characteristic overall shapes could be recognized and these were repeated, suggesting that the changes observed were cyclical, or pulsed with a frequency from *ca* 1–3 min. When the hyphal tip bumped into another hypha (Fig 9 at 576 s), the apex began to curve and extended parallel with the other hypha for some distance, until it eventually went below the obstructing hypha (Fig 9 at 624–992 s; Supplementary Material). Such interactions were common. Throughout the encounter the fluorescent patch maintained its position at the leading edge of the curving hyphal tip (Fig 9 at 576–672 s), but once the hypha was alongside, Spitzenkörper fluorescence shifted towards the other hypha (Fig 9 at 976 s and 992 s) and remained there until the end of observation. The region in the other hypha where the encounter occurred appeared depressed suggesting pressure from the extending tip (Fig 9 at 976–992 s). This may be followed until

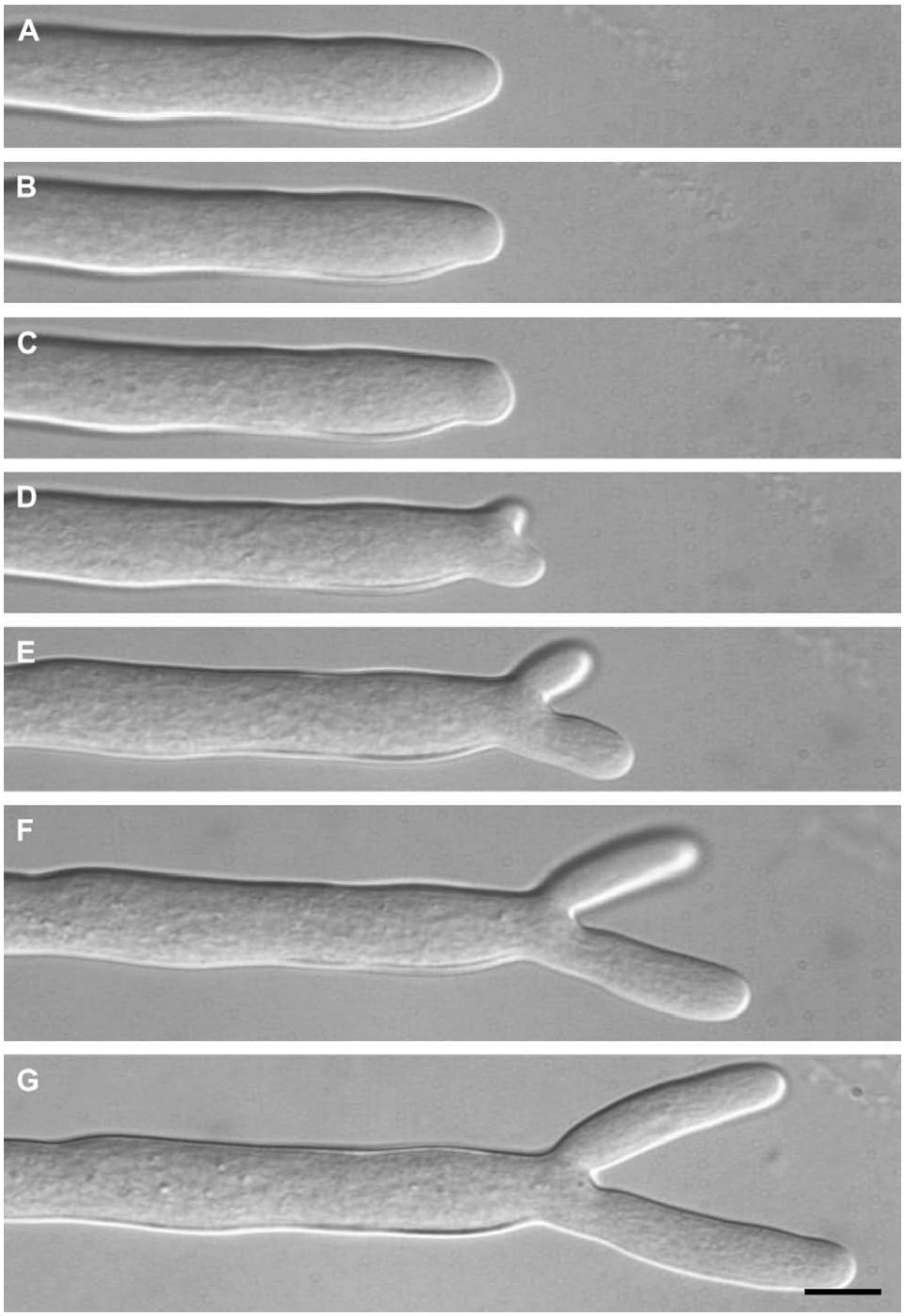

Fig 5 – Cessation of extension and change in the morphology of the apex (A–C) precede tip branching (D–G). Images taken at 10 min intervals. No septa can be seen at the branch point. Bar = 10 μm.

the end of the observations (Supplementary Material). In hyphal tips with light staining a correspondence in timing between appearance of fluorescence in the Spitzenkörper and the punctate fluorescence nearby could also be seen (Fig 10).

Ring-like structures

Also labelled with fluorescent carboxy-DFF were short tubular elements that formed characteristic ring-like structures (Figs 8–10; Supplementary Material). They were more obvious in continuously loaded cells but were also seen with pulse-chase loading at much lower levels of carboxy-DFF accumulation, though it is possible they were less frequent. They occurred throughout the hyphal tip cell but their interactions were easiest to observe in the sub-apical region, anterior to the more dense part of the vacuole system. These short tubular elements were often seen to occur in pairs and these pairs showed coordinated movements (Fig 8 at 96 s; Supplementary

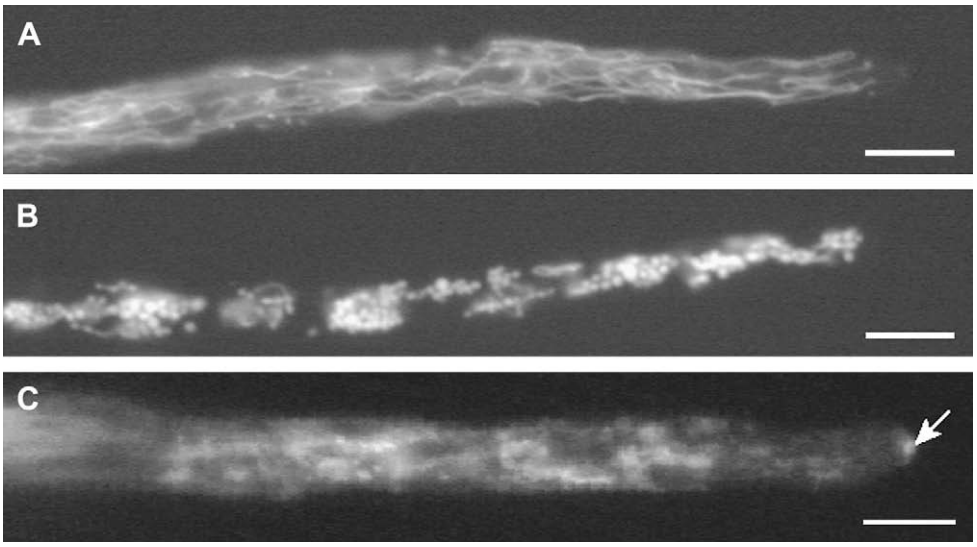

Fig 6 – Variation in vacuole form in different hyphal tips in slide culture. Two extremes of vacuole form are shown in A and B, from a tubular reticulum in association with only small vacuoles (A) to a system of essentially spherical larger vacuoles (B). In C the vesicle cloud of the Spitzenkörper has become well labelled (arrow). The tip region overall has a cloudy appearance thought to represent labelling in small vesicles. Bar = 10 μm.

Material). Several interacting pairs were seen in the sub-apical zone in continuously loaded cells (Figs 8, 9). The complexity of their interactions is shown in Figs 8–10. They underwent characteristic movements and shape changes, such that several distinct configurations could be recognized, the most obvious being a y-shaped structure with vesicle-like dilated tips (Fig 8 at 48–80 s), one or more rings (Fig 8 at 96–128 s, 160–208 s; Fig 9 at 576–656 s, 976–992 s; Fig 10 at 768 s, 960–992 s, 1072 s, 1152 s), a horseshoe shape (Fig 8 at 96 s) and an undulating tubule (Fig 8 at 160–208 s; Fig 9 at 576–592 s, 976–992 s). These configurations were observed as consecutive changes, and pairs were also seen to move in concert and to change their relative orientation, so that they frequently became oriented perpendicular to one another (Supplementary Material). Members of pairs also interacted with those of adjacent pairs, suggesting an interconnected system of similar units with a very complex activity pattern, but at least for the duration of our observations they remained in more or less the same cellular location (Figs 8, 9 at 160–992 s). Individual tubules from this system repeatedly made incursions from the sub-apical region into the apical dome (Fig 10) and were clearly seen in some instances to make transient contact with the fluorescent patch of vesicles. In the same hyphal tip, some frames (Supplementary Material) also show Spitzenkörper conformations that had a more strongly labelled inner region, adjacent to a central non-fluorescent core. In this hyphal tip, frames captured at 1328 and 1648 s show the tubule–Spitzenkörper interaction best, and other frames show tubules to contact (1024, 1040, 1184, 1313, 1680, 1744 s) or be in close proximity (1720, 1728, 1760 s) to the Spitzenkörper fluorescence. In earlier parts of the sequence the Spitzenkörper in the hyphal tip shown in Fig 10 was not yet strongly enough labelled for events in the apex to be clear. Other advancing hyphae in the movie showed similar incursions and contacts, but these are harder to decipher because of the much stronger punctate fluorescence in the tip. Ring-like structures were also seen in non-extending hyphae (not illustrated), where they were located closer to the apex.

Discussion

Radial colony growth in slide and Petri dish cultures and the effects of carboxy-DFFDA

Phanerochaete velutina grew as well in the slide culture system as on agar plates. Average colony extension rate of *Phanerochaete* in our standard Petri dish medium was 0.23 mm h^{-1}. This was very similar to the average colony growth rate of 0.24 mm h^{-1} found by Tlalka *et al.* (2002) and colony growth rate in the slide cultures was not significantly different from that on the agar plates. The cellophane sandwich culture system developed earlier for the study of organelle dynamics in *Pisolithus microcarpus* (Cole *et al.* 1997) did not give good results. *Phanerochaete velutina* grew but its mycelium was sparse, fragile and susceptible to disturbance when manipulated.

The lack of any effect of carboxy-DFFDA on individual tip extension rates was supported by lack of an effect on longer-term overall colony growth. This is in agreement with a general view that this probe at the concentration applied has low toxicity and supports its use in quantitative studies to investigate transport in the tubular vacuole system (Darrah *et al.* 2006).

Extension of Phanerochaete *hyphal tips under DIC optics in slide culture*

Slide culture is usually considered to be the method of choice for observation of relatively undisturbed and structurally well-preserved material and it has been used extensively in

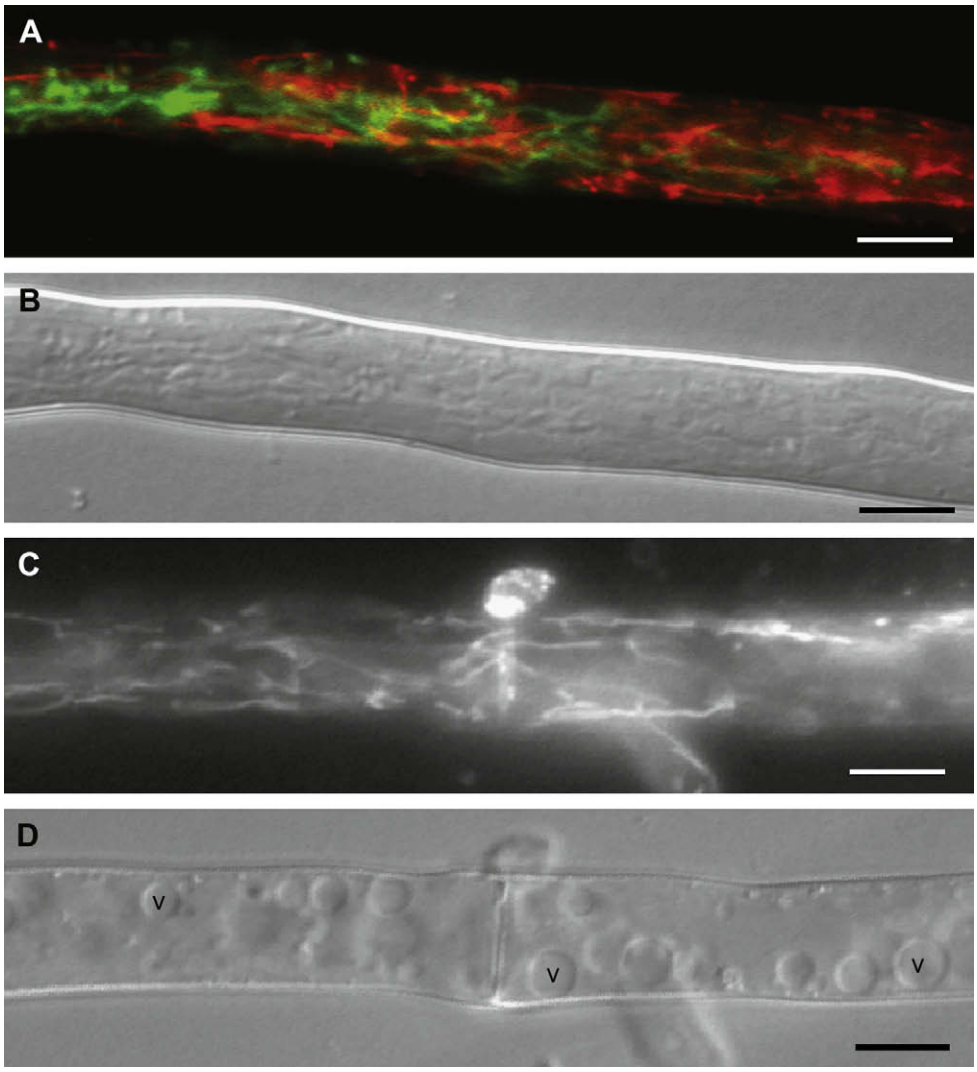

Fig 7 – (A) Part of a hyphal tip cell showing dual-localisation of the vacuole system with CMAC (green pseudo-colour) and mitochondria with $DiOC_6(3)$ (red pseudo-colour) and (B) the corresponding DIC image. (C) $DiOC_6(3)$ labelled mitochondria crossing the septum between the apical and penultimate cell and (D) the corresponding DIC image showing that the vacuoles (v) are spherical in both these cells. Bar = 10 μm.

the study of micro-organisms including filamentous fungi. The approach dates back to Brefeld (1877), and there are many protocols (e.g. Riddell 1950; Hofherr 1978; Onions *et al.* 1981). The system used here resembles that described by Aist (1969). Like all such methods, it is based on the principle of incubation in a moist chamber and minimum disruption on sampling. It has a number of advantages for microscopic work compared with the other methods we tried. The hyphal tips grew in a narrow plane, so they could be easily focused and observed under the microscope. The thin layer of agar coating the slide did not create problems either in labelling hyphae with small amounts of probe, or in producing images with DIC and epifluorescence optics. Though disturbance was minimal, reducing the confounding effects of cutting, lifting and drying out on hyphal tip extension and behaviour, many hyphae exhibited a series of bulges consistent with the effects of minor disturbance on tip morphology, as described in *Rhizoctonia solani* by Bartnicki-García *et al.* (1995), and this is probably accompanied by changed Spitzenkörper behaviour (*cf.* Reynaga-Peña & Bartnicki-García 2005), but this was not investigated here. There was also an initial period of very slow extension. An initial 60 min acclimation period was necessary to allow the hyphae time to stabilise/habituate to a constant extension rate before treatment and observation, but this clearly must be experimentally determined according to fungal species and conditions. Further improvements could be made by using a well slide and reducing the thickness of the agar. There were no obvious effects of irradiation by the microscope lamps on extension and once habituated the hyphal tips continued to extend for many hours at constant rates, provided that water was regularly added. This provides opportunities for sustained observation. The method has many of the advantages of the cellophane sandwich technique (Cole *et al.* 1997) without the problems of low oxygen tension and the potential for damage or loss of material when the cellophane is removed and material cut and

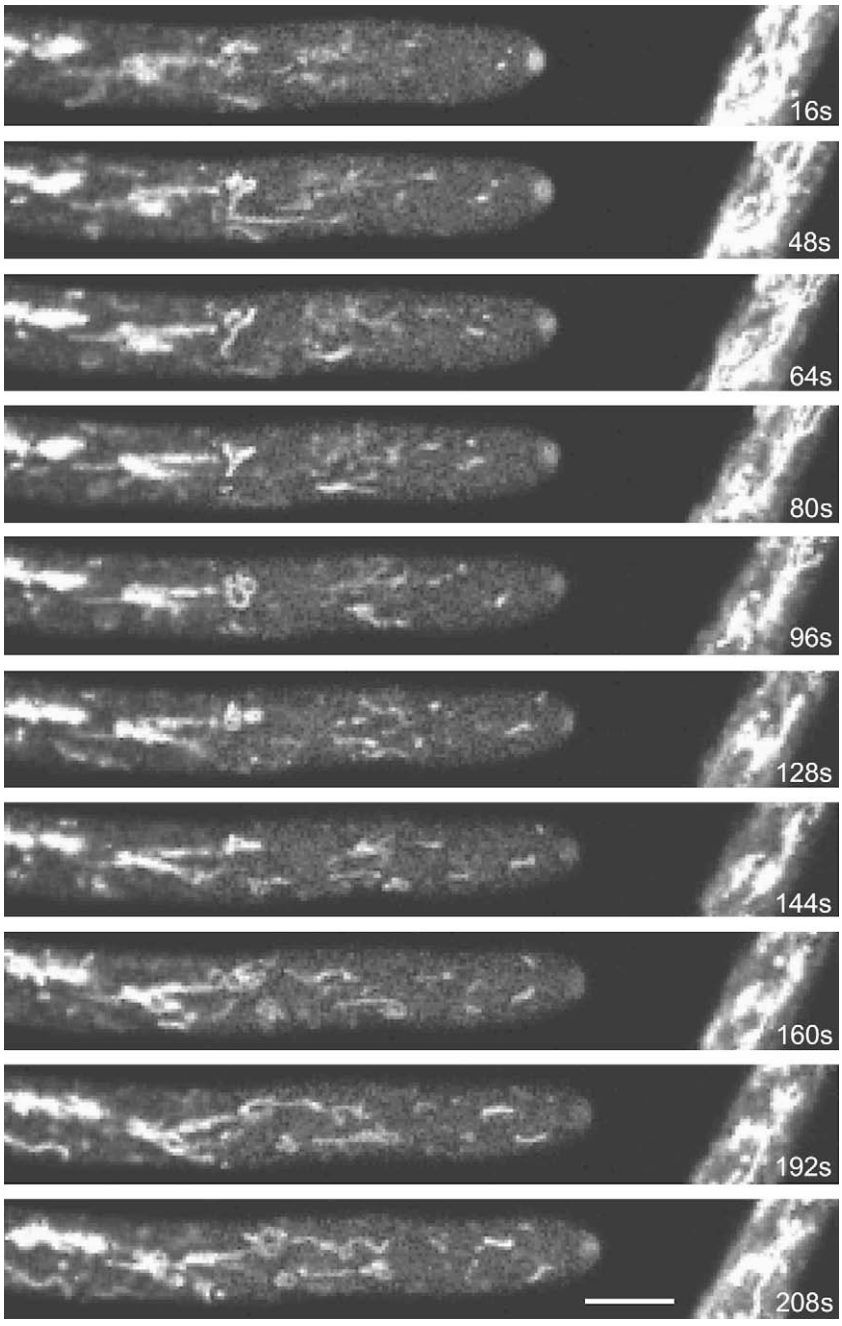

Fig 8 – Confocal microscopy to show Spitzenkörper transformations, very small vesicles, and tubules that change orientation and produce ring-like structures in a hyphal tip continuously loaded with carboxy-DFFDA. Maximum *x,y,z* projections taken from an *x,y,z,t* sequence (shown as a video in the Supplementary Material). Times are indicated in seconds from a series lasting 1920 s. The extension rate was 2.3 μm min^{-1}. Note pairing of ring-like structures and their interactions with other similar tubules and ring-like structures. Bar = *ca* 10 μm.

transferred to a slide. It should be stressed that whatever the protocol, it is essential to pay attention to detail with respect to exact reproducibility of procedures and timing as found in earlier work (e.g. Howard & Aist 1977).

Labelling of vacuoles and mitochondria

The vacuole system stained well with both carboxy-DFFDA and CMAC. A similar accumulation of CMAC, as well as CMFDA, another chloromethyl thiol tracker reagent, also occurred in *Pisolithus microcarpus*, formerly named *P. tinctorius* (Cole *et al.* 1997). This further supports a role for vacuoles in detoxification processes in fungi. CMAC is a thiol-reactive cell tracker reagent and its accumulation in vacuoles is consistent with the presence of intracellular thiols and a glutathione-mediated transport pathway to the vacuole. This fungal glutathione-dependent detoxification pathway appears to be similar to the better-studied pathway in plant cells (Coleman

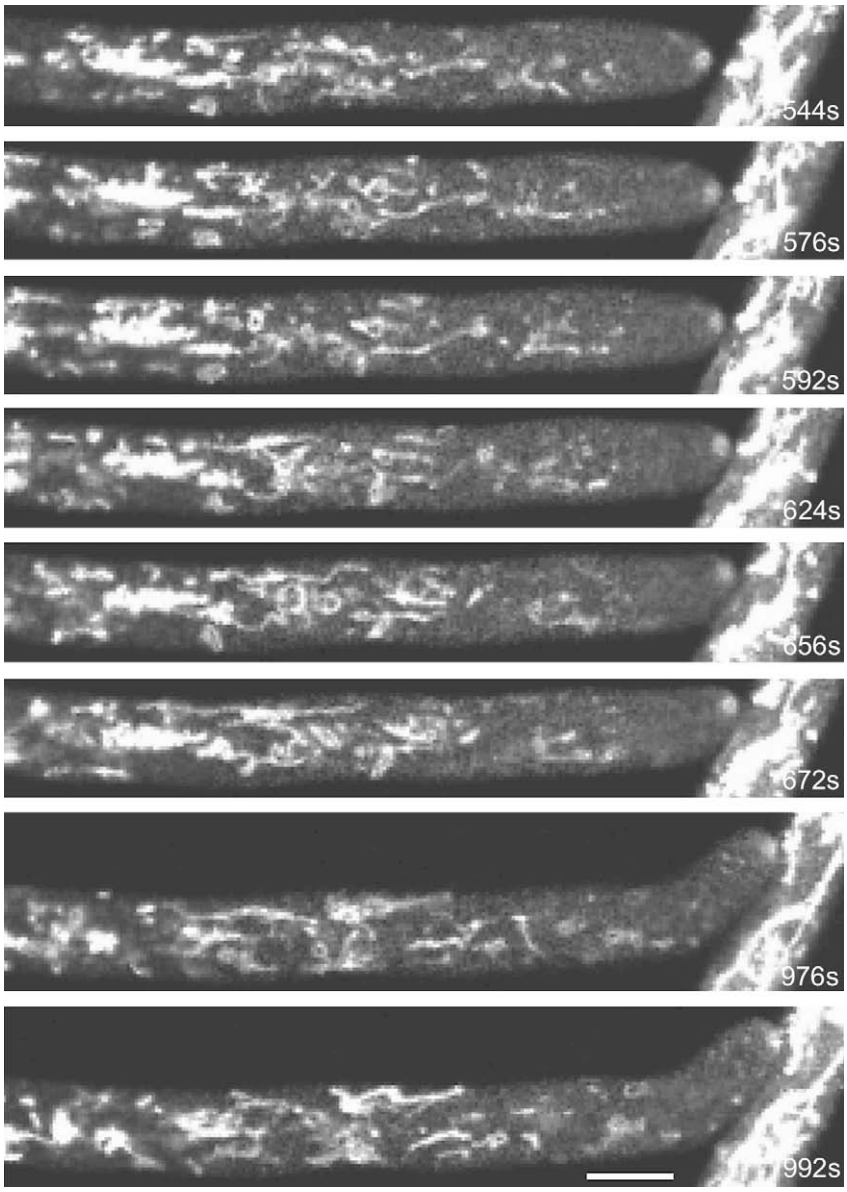

Fig 9 – Confocal images of the same hyphal tip as Fig 8 later in the sequence as the tip approaches and bumps into another hypha (see also Supplementary Material). Before contact (544 s) the Spitzenkörper is central and has a dark non-fluorescent core. At contact (576 s) the Spitzenkörper is still at the leading edge of the apex, but by 592 s it has begun to move laterally. By 624 s this is more pronounced and the tip is flattened against the obstacle hypha. Flattening becomes more pronounced (656 s, 672 s) and a new direction of extension is established with the Spitzenkörper at the leading edge. The hypha extends parallel to the obstacle hypha with the Spitzenkörper displaced towards it (976 s, 992 s) and the surface of the latter shows indentation with very close contact at the Spitzenkörper. The number and staining intensity of tubules and ring-like structures has increased in the subapical region and they show multiple interactions. Bar = *ca* 10 μm.

et al. 1997; Clemens & Simm 2003; Pócsi *et al.* 2004). Preliminary observations indicate that the form of the vacuole system was related to extension rate and branching. Rapidly extending tips were usually dominated by a fine tubular reticulum of vacuoles while slowly-extending hyphae contained larger, less motile spherical vacuoles. The change in the vacuole system towards a reticulum that occurs prior to side branch emergence is one of the few observable changes in hyphae that herald lateral branching (*cf.* Riquelme & Bartnicki-Garcia 2004).

Continuity of tubular vacuoles across septa was not specifically investigated here, but has been reported in *Pisolithus* by Shepherd *et al.* (1993) and in *Phanerochaete* by Darrah *et al.* (2006). In both species repeated incursions of vacuolar tubules through the septal pore into the adjacent cell were recorded by confocal microscopy. The invading tubules frequently fused with vacuoles of the recipient cell, providing a mechanism for intercellular transfer of membrane and vacuolar content. Our observations that mitochondria, stained with $DiOC_6(3)$, formed a reticulum and were frequently found across mature septa

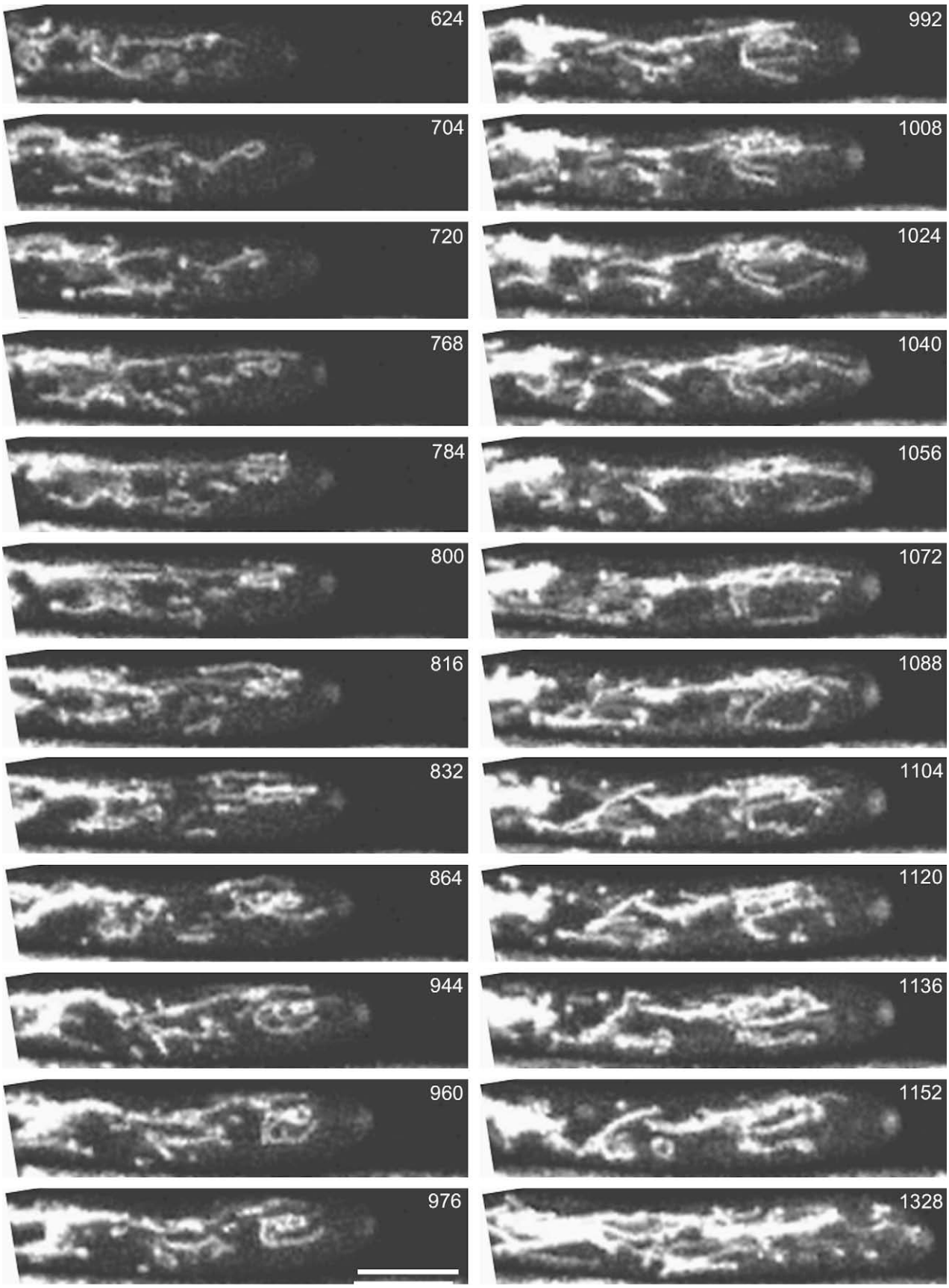

Fig 10 – Confocal images of another lightly labelled hyphal tip to show contact between ring-like structures and Spitzenkörper. The patch of Spitzenkörper fluorescence maintains its position at the hyphal apex throughout the series and xbecomes more obvious as staining intensifies. A tubule undergoes complex morphological changes to appear as a ring and branches (624–992 s) one of which extends to contact the Spitzenkörper patch (992–1040 s) and then withdraws (1056 s). Another contact is made at 1328 s. (From the same sequence: see Supplementary Material). Bar = *ca* 10 μm.

indicate that potentially they, like vacuoles, are a conduit for intercellular transport. There is the potential to maintain mitochondrial continuity from cell to cell, controlled by transient closure of the pore as found for tubular vacuole systems (cf. Darrah *et al.* 2006). Considering the unique content and non-nuclear inheritance of mitochondria, movement of mitochondrial content along the hyphal apex by processes such as diffusion, within its own compartment, has distinct benefits. It would enable equilibration of mitochondrial content (substrates, products, and enzymes) around an elongated cell without the necessity for either fission and fusion or organelle propulsion.

Labelling of Spitzenkörper and vesicles

Carboxy-DFF, the fluorescent hydrolysis product of carboxy-DFFDA, accumulates in the lumen of compartments where membrane integrity is not compromised. Carboxy-DFFDA itself is not fluorescent but, like other carboxy-fluorescein derivatives, is thought to be hydrolysed by cytoplasmic esterases to release a fluorescent product that is rapidly transported across vacuole membranes and trapped inside the vacuoles (Shepherd *et al.* 1993; Cole *et al.* 1997, 1998). The finely punctate fluorescence in the apical region of continuously-loaded hyphal-tip cells of *Phanerochaete velutina* is thought to represent probe accumulated in very small vesicles that are at or below the resolution of the light microscope. It contrasts with the diffuse uniform cytoplasmic fluorescence typically seen in 6-carboxy-fluorescein loaded hyphal tip cells of other species treated with anion transport inhibitors such as probenecid (Cole *et al.* 1997, 1998). Under continuous carboxy-DFFDA labelling the hyphal tips continued to extend at rapid, undiminished rates. The strong staining of very small vesicles in *Phanerochaete* under these conditions, but not in some other fungi (Cole *et al.* 1997), may be attributed to its very rapid extension and turnover, and/or the continuous loading allowing continued accumulation of hydrolysis product to high levels, so that there is sufficient for it to be visible in very small structures.

The more intense fluorescent patch at the apex (*cf.* Inselman *et al.* 1999) is also thought to represent accumulation of probe in vesicles, in this case in the vesicle cloud that constitutes part of the Spitzenkörper (see for example Howard 1981; Roberson & Fuller 1988; Bourett & Howard 1994; Bourett *et al.* 2007). This interpretation is based on the mechanism of 'staining' described above in conjunction with its location at the apex of extending hyphae, its loss when the hyphae stop extending, and the observed shifts in its position in concert with changes in direction of extension. These mimic the appearance and positional shifts seen when the Spitzenkörper is labelled and/or observed in a range of fungi by other methods (*cf.* Bartnicki-García *et al.* 1995; Lopéz-Franco & Bracker 1996; Read *et al.* 1998; Fischer-Parton *et al.* 2000; Harris *et al.* 2005; Reynaga-Peña & Bartnicki-García 2005; Araujo-Palomares *et al.* 2007; Read 2007; Valkonen *et al.* 2007). Labelling of the Spitzenkörper and other small vesicles with carboxy-DFF indicates that either a transport pathway connects vacuoles with at least a subset of Spitzenkörper vesicles, or alternatively that there is a mechanism for accumulation of probe across vesicle membranes, as there is for vacuoles. Because of their small size and the limits to resolution, it is not possible to say which subset of Spitzenkörper vesicles is labelled. The data show that carboxy-DFFDA is an attractive alternative to FM4-64 for studying Spitzenkörper vesicle dynamics in this fungus, labelling the lumen rather that the membrane. Changes in level of fluorescence within the Spitzenkörper patch during sequences may be attributed to changes in vesicle numbers or their labelling.

The incursion of tubules both from the vacuolar reticulum (see Darrah *et al.* 2006 supplemental file 1 Fig 2c video. mov.) and from ring-like structures into the apical dome, and their contact with Spitzenkörper fluorescence, indicate the potential of tubules rather than vesicles to deliver material directly to the apex. These tubular incursions into the apex are transient and are not readily identified in single plane or small z-dimension confocal microscopy. Riquelme & Bartnicki-García (2008) have suggested that longitudinal transport of chitin synthase-GFP constructs to the apical region is not by the conventional Golgi secretory pathway or the endocytic route, but could be *via* tubulo-vesicular processes from the tubular vacuole system. This is based on the observations that in their constructs fluorescence is localised in membranous structures of various shapes from spheres to elongated tubes. The images of CHS-6-GFP distribution in *Neurospora* hyphae (Riquelme *et al.* 2007) bear a striking resemblance to those of tubular vacuolar systems in various hyphal regions, when viewed in a similar way in *Phanerochaete* and other fungi. In their model Riquelme & Bartnicki-García (2008) suggest that as the tubular vacuoles move forward, keeping pace with the advancing tip, they gradually decrease in size, until dispersing into very fine vesicles that accumulate in the Spitzenkörper. We have not found any evidence of disintegration of the tubular vacuolar system in the sub-apical region in *Phanerochaete* or other fungi. Another possibility supported by the movements of tubules presented here is that tubules from the vacuolar reticulum deliver material directly to the Spitzenkörper region. We speculate that if these tubules do release material in vesicles, this is likely to take place at or very close to the Spitzenkörper.

Ring-like structures

Ring-like configurations of tubules that accumulate carboxy-DFF have been described previously in the literature both in *Pisolithus* and *Phanerochaete* (Cole *et al.* 1997; Darrah *et al.* 2006), and are very common (A.E.A., unpubl. obs.). They are usually considered to be part of the tubular vacuole system. Ohneda *et al.* (2002) also reported ring-like structures arising from tubules in cells of an *Aspergillus oryzae* transformant expressing carboxypeptidase Y-enhanced green fluorescent protein (CPY-EGFP) fusion protein under certain growth conditions. The expression of this construct suggested that ring-like structures are related to vacuoles, but differ from typical vacuoles in that they do not accumulate CMAC. Ohneda *et al.* (2002) suggested that they might be organelles in the secretory pathway to vacuoles. Ring-like structures were also demonstrated by CPY-EGFP fluorescence in two *A. oryzae* mutants (dfc-14 and hfm-4) that mislocalise this protein so that it is secreted rather than accumulating in mature vacuoles, and they were tentatively identified as equivalent to the deformed pre-vacuolar (late endosomal) compartment seen in class E *vps* mutants of *Saccharomyces cerevisiae* (Ohneda *et al.* 2005). Ring-like structures have also been described in several yeast mutants deficient in ER to Golgi, or endosome to vacuole,

transport where they are considered to represent endosomes or Golgi bodies with abnormal morphologies (Gaynor *et al.* 1998; Kranz *et al.* 2001). Our data with carboxy-DFFDA indicate that the ring-like structures in *Phanerochaete* are not unusual or abnormal structures, nor are they found only under specific growth conditions, but are regular components of extending and non-extending hyphae with normal morphology. Some of them are shown by Darrah *et al.* (2006) in their Fig 2C–D and videos S2C–D. Darrah *et al.* (2006) described them as 'transiently isolated tubules' that 'rearranged to form branched, Y-shaped structures or loops or collapsed back to form vesicles'. Our observations further indicate that these paired structures can occur as an interacting group, and their behaviour suggests that they may be sorting structures. It is not clear at this stage whether they are a distinct subcompartment of the vacuole system or whether they are some other organelle. They appear to be too large for Golgi bodies. Late endosomal compartments visualized by expressed constructs of EGFP and compartment markers in non-mutants of other filamentous fungi such as *A. oryzae* are reported to be punctate structures (Tatsumi *et al.* 2007). Looking at the complexity of ring-like structures, and their interactions and shape changes over short time periods, their ultrastructural characterization would be difficult. Similarly, they may not be recognized as they would mostly appear like vesicles in single plane confocal microscopy, the method commonly used to identify target organelles in work with GFP constructs. However, targeting organelles by expression of GFP constructs with gene products of known localization in conjunction with 4-D confocal microscopy provides a potential means for their identification.

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Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mycres.2008.11.014.

REFERENCES

Aist JR, 1969. The mitotic apparatus in fungi, *Ceratocystis fagacearum* and *Fusarium oxysporum*. *Journal of Cell Biology* **40**: 120–135.

Allaway WG, Ashford AE, Heath IB, Hardham AR, 1997. Vacuolar reticulum in oomycete hyphal tips: an additional component of the Ca^{2+} regulatory system? *Fungal Genetics and Biology* **22**: 209–220.

Araujo-Palomares CL, Castro-Longoria E, Riquelme M, 2007. Ontogeny of the Spitzenkörper in germlings of *Neurospora crassa*. *Fungal Genetics and Biology* **44**: 492–503.

Ashford AE, Allaway WG, 2007. Motile tubular vacuole systems. In: Howard RJ, Gow NAR (eds), *The Mycota. VIII. Biology of the Fungal Cell*. 2nd edn. Springer-Verlag, Berlin, pp. 49–86.

Bartnicki-García S, Bartnicki DD, Gierz G, López-Franco R, Bracker CE, 1995. Evidence that Spitzenkörper behavior determines the shape of a fungal hypha: a test of the hyphoid model. *Experimental Mycology* **19**: 153–159.

Bebber DP, Tlalka M, Hynes J, Darrah PR, Ashford A, Watkinson SC, Boddy L, Fricker MD, 2007. Imaging complex nutrient dynamics in mycelial networks. In: Gadd GM, Watkinson SC, Dyer PS (eds), *Fungi in the Environment*. Cambridge University Press, Cambridge, pp. 1–21.

Bourett TM, Howard RJ, 1994. Enhanced labeling of concanavalin A binding sites in fungal endomembranes using a double-sided, indirect method. *Mycological Research* **98**: 769–775.

Bourett TM, James SW, Howard RJ, 2007. The endomembrane system of the fungal cell. In: Howard RJ, Gow NAR (eds), *The Mycota. VIII. Biology of the Fungal Cell*. 2nd edn. Springer-Verlag, Berlin, pp. 1–47.

Brefeld O, 1877. *Botanische Untersuchungen über Schimmelpilze. III. Heft: Basidiomyceten I*. Verlag Von Arthur Felix, Leipzig.

Clemens S, Simm C, 2003. *Schizosaccharomyces pombe* as a model for metal homeostasis in plant cells: the phytochelatin-dependent pathway is the main cadmium detoxification mechanism. *New Phytologist* **159**: 323–330.

Cole L, Hyde GJ, Ashford AE, 1997. Uptake and compartmentalisation of fluorescent probes by *Pisolithus tinctorius* hyphae: evidence for an anion transport mechanism at the tonoplast but not for fluid-phase endocytosis. *Protoplasma* **199**: 18–29.

Cole L, Orlovich DA, Ashford AE, 1998. Structure, function, and motility of vacuoles in filamentous fungi. *Fungal Genetics and Biology* **24**: 86–100.

Coleman JOD, Blake-Kalff MMA, Davies TGE, 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science* **2**: 144–151.

Darrah PR, Tlalka M, Ashford A, Watkinson SC, Fricker MD, 2006. The vacuole system is a significant intracellular pathway for longitudinal solute transport in basidiomycete fungi. *Eukaryotic Cell* **5**: 1111–1125.

Fischer-Parton S, Parton RM, Hickey PC, Dijksterhuis J, Atkinson HA, Read ND, 2000. Confocal microscopy of FM4-64 as a tool for analyzing endocytosis and vesicle trafficking in living fungal hyphae. *Journal of Microscopy* **198**: 246–259.

Gaynor EC, Chih-Ying C, Emr SD, Graham TR, 1998. ARF is required for maintenance of yeast Golgi and endosome structure and function. *Molecular Biology of the Cell* **9**: 653–670.

Harris SD, Read ND, Roberson RW, Shaw B, Seiler S, Plamann M, Momany M, 2005. Polarisome meets Spitzenkörper: microscopy, genetics, and genomics converge. *Eukaryotic Cell* **4**: 225–229.

Hofherr L, 1978. A new technique for the preparation of permanent reference cultures of fungi. *Canadian Journal of Microbiology* **24**: 1275–1277.

Howard RJ, 1981. Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkörper, cytoskeleton and endomembranes after freeze substitution. *Journal of Cell Science* **48**: 89–103.

Howard RJ, Aist JR, 1977. Effects of MBC on hyphal tip organization, growth, and mitosis in *Fusarium acuminatum*, and their antagonism by D_2O. *Protoplasma* **92**: 195–210.

Inselman AL, Gathman AC, Lilly WW, 1999. Two fluorescent markers identify the vacuolar system of *Schizophyllum commune*. *Current Microbiology* **38**: 295–299.

Kranz A, Kinner A, Kölling R, 2001. A family of small coiled-coil-forming proteins functioning at the late endosome in yeast. *Molecular Biology of the Cell* **12**: 711–723.

Lopéz-Franco R, Bracker CE, 1996. Diversity and dynamics of the Spitzenkörper in growing hyphal tips of higher fungi. *Protoplasma* **195**: 90–111.

Ohneda M, Arioka M, Kitamoto K, 2005. Isolation and characterization of *Aspergillus oryzae* vacuolar protein sorting mutants. *Applied Environmental Microbiology* **71**: 4856–4861.

Ohneda M, Arioka M, Nakajima H, Kitamoto K, 2002. Visualization of vacuoles in *Aspergillus oryzae* by expression of CPY-EGFP. *Fungal Genetics and Biology* **37**: 29–38.

Onions AHS, Allsop D, Eggins HOW, 1981. *Smith's Introduction to Industrial Mycology*, 7th edn. Edward Arnold, London.

Pócsi I, Prade RA, Pennickx MJ, 2004. Glutathione, altruistic metabolite in fungi. *Advances in Microbiological Physiology* **49**: 1–76.

Read N, 2007. Environmental sensing and filamentous fungal lifestyle. In: Gadd G, Watkinson SC, Dyer PS (eds), *Fungi in the Environment*. Cambridge University Press, Cambridge, pp. 38–57.

Read ND, Fischer S, Parton RM, 1998. Extended summary Physicochemical and Biophysical Panel of the SCI Pesticides Group and the Royal Microscopical Society meeting: microlocalisation of chemicals within biological systems. *Pesticide Science* **54**: 179–181.

Rees B, Shepherd VA, Ashford AE, 1994. Presence of a motile tubular vacuole system in different phyla of fungi. *Mycological Research* **98**: 985–992.

Reynaga-Peña CG, Bartnicki-García S, 2005. Cytoplasmic contractions in growing fungal hyphae and their morphogenetic consequences. *Archives of Microbiology* **183**: 292–300.

Riddell RW, 1950. Permanent stained mycological preparations obtained by slide culture. *Mycologia* **42**: 256–270.

Riquelme M, Bartnicki-García S, 2004. Key differences between lateral and apical branching in hyphae of *Neurospora crassa*. *Fungal Genetics and Biology* **41**: 842–851.

Riquelme M, Bartnicki-García S, 2008. Advances in understanding hyphal morphogenesis: ontogeny, phylogeny and cellular localization of chitin synthases. *Fungal Biology Reviews* **22**: 56–70.

Riquelme M, Bartnicki-García S, González-Prieto JM, Sánchez-Léon E, Verdín-Ramos JA, Beltrán-Aguilar A, Freitag M, 2007. Spitzenkörper localization and intracellular traffic of green fluorescent protein-labeled CHS-3 and CHS-6 chitin synthases in living hyphae of *Neurospora crassa*. *Eukaryotic Cell* **6**: 1853–1864.

Roberson RW, Fuller MS, 1988. Ultrastructural aspects of the hyphal tip of *Sclerotium rolfsii* preserved by freeze substitution. *Protoplasma* **146**: 143–149.

Shepherd VA, Orlovich DA, Ashford AE, 1993. Cell-to-cell transport via motile tubules in growing hyphae of a fungus. *Journal of Cell Science* **105**: 1173–1178.

Sudbery P, Court H, 2007. Polarised growth in fungi. In: Howard RJ, Gow NAR (eds), *The Mycota. VIII. Biologyof the Fungal Cell*. 2nd edn., Springer-Verlag, Berlin, pp. 137–166.

Tatsumi A, Shoji J, Kikuma T, Arikola M, Kitamoto K, 2007. Aggregation of endosomal vacuolar compartments in the Aovps24-deleted strain in the filamentous fungus *Aspergillus oryzae*. *Biochemical and Biophysical Research Communications* **362**: 474–479.

Tlalka M, Watkinson SC, Darrah PR, Fricker MD, 2002. Continuous imaging of amino-acid translocation in intact mycelia of *Phanerochaete velutina* reveals rapid, pulsatile fluxes. *New Phytologist* **153**: 173–184.

Valkonen M, Kalkman ER, Saloheimo M, Penttilä M, Read ND, Duncan RR, 2007. Spatially segregated SNARE protein interactions in living fungal cells. *Journal of Biological Chemistry* **282**: 22775–22785.

Watkinson SC, Boddy L, Burton K, Darrah PR, Eastwood D, Fricker MD, Tlalka M, 2005. New approaches to investigating the function of mycelial networks. *Mycologist* **19**: 11–17.