THE RESPONSE OF THE VACUOLAR RETICULUM OF SAPROLEGNIA FERAX TO THE PRESENCE OF GTPASE MODULATOR, GTPγ-S

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Abstract

Modulating GTPase with GTPγ-S resulted in localised changes to the vacuolar reticula of *S. ferax* hyphae. The response to GTPγ-S implies that the reticula perform different functions in different regions of the hyphae. Results from this study suggest that the vacuolar reticulum may facilitate membrane trafficking along the length of the hypha to the tip. This is likely to involve sequestration of nascent membrane in the subapical and basal region followed by deposition in the plasma membrane of the apical region. The accumulation of metabolites or luminal content in the vacuolar reticula of GTPγ-S treated hyphae also implies a role of the reticulum in intracellular metabolite trafficking.

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Introduction

Relatively little is known about the mechanisms regulating hyphal growth in species of the genus *Saprolegnia*, Phylum Oomycota. The hyphae of species of *Saprolegnia* are structurally different from that of filamentous fungi (Hyde and Heath 1997, Levina *et al.* 1995). In contrast to the dynamic extension of tubules with interconnected clusters of vacuoles at the tip of fungal hypha (Rees *et al.* 1994, Shepherd *et al.* 1993), Oomycetes have fine, interconnected, static tubules extending towards the tip (Allaway *et al.* 1997, Lilje 2004). The presence of a fine reticulate structure at the apex of *Saprolegnia* hyphae suggests that the structure is involved in growth (Allaway *et al.* 1997). The reticulum appears to expand while maintaining contact with a large vacuole in the basal region of the hypha (Allaway *et al.* 1997). Its close association with the plasma membrane in the apical region and its predominantly tubular endomembrane compartmentalisation suggests that the efficient transfer of membrane to the apical region of growing hyphae involves a tubular transport mechanism (Lilje 2004, Mironov *et al.* 1997). In contrast, the dynamic tubular extensions found in filamentous fungi (Hyde *et al.* 2002) have been associated with a vesicular transport mechanism that allows continuous and efficient transfer of nascent cell wall membrane from the parent spherical vacuole in the subapical region to the growing apical region (Garrill *et al.* 1992). Whether vesicles facilitate membrane transfer in species of *Saprolegnia* is not known. Modulating the transfer of membrane between the static tubules and the plasma membrane will help to clarify the membrane transport mechanism that operates in *Saprolegnia* species.

GTP binding proteins (GTPase) regulate the interchangeable vesicle tubule forms observed in animal and plant cells and fungi (Gilman 1987, Hyde, *et al.* 2002, Takei *et al.* 1995, Takai *et al.* 2001). It is likely that because GTPases control many aspects of cell physiology, including membrane fission or vesiculation by hydrolysing GTP to GDP (Alberts *et al.* 2002, Gilman 1987, Hyde *et al.* 2002, Takei *et al.* 1995, Takai *et al.* 2001); that they will influence membrane deposition during hyphal growth. There are three types of GTPase, monomeric (18-33kDa); heterotrimeric G-protein (39-52kDa); and large GTP-binding protein (50kDa and higher) (Alberts *et al.* 2002). Monomeric and large GTPase are of

particular interest because of their association with vesicle trafficking in fungi (Takai *et al.* 2001). Large GTP-binding proteins such as self-assembling tubulin and dynamin, are involved in membrane extension and vesicle formation respectively (Alberts *et al.* 2002, Hyde *et al.* 2002). Modulators such as guanosine 5'-O-(3-thiotriphosphate) (GTPγ-S) alter GTPase activity. GTPγ-S is a non-specific, non-hydrolysable analogue of GTP (Gilman 1987). In filamentous fungi it has been noted that GTPγ-S enhances tubulation in the hyphal tip while inhibiting vesicle formation in filamentous fungi (Hyde *et al.* 2002).

It is hypothesised that membrane trafficking in the apical region of growing *S. ferax* hyphae involves GTPase activity. As a consequence, the presence of GTPγ-S will result in changes to the vacuolar reticulum. This study aims to clarify the transport system associated with membrane deposition in *S. ferax*. The evidence will either support the concept of a tubular or vesicular transport mechanism in *S. ferax* tip growth.

Materials and Methods

Maintenance of *S. ferax* culture

The method outlined in Allaway *et al.* (1997) was used in the preparation of *S. ferax* (Gruith.) Thuret. *Saprolegnia ferax* (H302) was obtained from stock cultures kept in the Research School of Biological Sciences, Canberra. In brief, the cultures were initially grown on GYPS agar (Beakes and Gay 1981) at 23°C in darkness until the plates were confluent. Small agar cubes of culture were sectioned from the leading edge of the colony and incubated in GYPS liquid medium (GYPSL) [5g D-glucose, 0.5g peptone, 0.5gKH_2PO_4, 0.15g $MgSO_4.7H_20$ and 0.05g yeast extract in 1L dH_20]. Hyphal segments (approx. 0.2 mm in length) were cut aseptically and incubated overnight at 23°C in GYPSL in sterile watch glasses to form cultures.

In an experiment fluorescent and electron microscope observations were made. An experiment consisted of eight GTPγ-S treated and four untreated *S. ferax* cultures. The experiment was replicated four times.

Fluorescent vacuolar reticulum staining and GTPγ-S treatment

After overnight incubation at 23°C, six cultures of *S. ferax* were incubated with 20μg/ml Oregon Green 488 carboxylic acid diacetate (carboxy-DFDDA) (Molecular Probes, Eugene, OR) for selective staining of the vacuolar reticulum at 23°C for 40 min. Carboxy-DFFDA selectively labels the vacuoles by being hydrolysed in the cytoplasm and sequestered in anionic form across the tonoplast (Cole *et al.* 1998, Hyde *et al.* 2002). The cultures were then treated with 100 μM GTPγ-S (Molecular Probes) for 20 (n = 2) or 40 mins (n = 2) or with no GTPγ-S (n = 2) before being mounted onto slides.

The cultures were mounted by gently sliding a coverslip under each culture to lift the culture. The coverslip was then inverted onto a slide over teflon spacers (Heath 1987, Heath 1988) so that hyphae were not squashed. Viability of the cells was confirmed by the presence of cytoplasmic streaming, an undisturbed vacuolar reticulum and general hyphal morphology (Allaway *et al.* 1997, Rees *et al.* 1994).

GTPγ-S treatment and preparation for Transmission electron microscope (TEM)

After overnight incubation at 23°C, six cultures of *S. ferax* were treated with 100 μM GTPγ-S (Molecular Probes) for 20 (n = 2) or 40 mins (n = 2) or with no GTP-γ-S (n = 2). Cultures were then fixed in 2% glutaraldehyde (ProSciTech, Australia) in 0.1M phoshate buffer [39ml (1.39g $NaH_2PO_4.H_20$/100ml $d.H_2O$) and 61ml (1.42g Na_2HPO_4/100ml $d.H_2O$)], pH 7.0 at 23°C for 60 min. The fixed cultures were then washed three times for 15 mins each with deionised H_2O at 23°C and postfixed in 2% OsO_4 (ProSciTech, Australia) in 0.1M phosphate buffer for 60 mins. Cultures were then washed three times as previously described, then dehydrated in 30% acetone for 15 mins followed by an equivalent time in 50, 70, 96 and 100% acetone. Acetone was removed from the cultures by continuous rotation in 1:3 Spurr (ProSciTech, Australia) in acetone (Sigma) at 23°C overnight followed by 2:3 Spurr in acetone and 100% Spurr for an equivalent time and conditions. Cultures were then orientated for embedding in Spurr in a flat mould and polymerised overnight. Flat embedded segments are difficult to manipulate and therefore were cut after polymerisation and placed in a capsule with additional Spurrs for further polymerisation overnight. Ultrathin sections (80 μm) were cut using the Leica Ultracut UCT and placed on prepared grids (ProSciTech, Australia). 100 mesh grids (ProSciTech, Australia) were prepared by covering them with a film of 0.6% formvar (ProSciTech, Australia) followed by carbon coating. Grids containing sections were then incubated in 2% uranyl acetate (BDH) for 6 mins, rinsed three times with dH_2O, incubated in lead citrate (AJAX Chemicals) for 6 mins and rinsed three times with dH_2O.

Images from *S. ferax* hyphae prepared by the chemical fixation protocol described above were

compared with images obtained from propane plunge fixed specimens and other studies on *S. ferax* (Cole *et al.* 2000, Gay *et al.* 1971, Heath *et al.* 1971). The organelles, cell membrane, vesicles and vacuolar reticulum were found to be comparable. Changes to the ultrastructure, in particular the vacuolar reticulum of treated *S. ferax* hyphae were consistently replicated in different specimens and demonstrated a graded response correlating to treatment time. The glutaraldehyde fixation, osmium post-fixation and Spurr embedding protocol was also used because it allowed for specimen orientation for longitudinal and transverse sectioning of hyphae. Ultrastructural features of *S. ferax* hyphae observed in the current study were confirmed by comparing them with published images (Cole *et al.* 2000, Gay *et al.* 1971, Heath *et al.* 1971).

Determining growth over time of control and GTPγ-S treated cultures

Eight *S. ferax* hyphae segments (approx. 0.2 mm in length) were cut aseptically and incubated in sterile watch glasses containing GYPSL overnight at 23°C. Cultures were then randomly selected and equally distributed to either the control (GYPSL) or the treatment group (100 µM GTPγ-S). Each culture was mounted onto a slide by gently sliding a coverslip under the culture to lift it out of the watch glass. The coverslip was then inverted onto a drop of either GYPSL or 100 µM GTPγ-S on a slide. Teflon spacers were positioned on either side of the drop of GYPSL or 100 µM GTPγ-S to prevent physical restrictions to the growth of the hypha. The distance grown by three or four randomly selected hyphae for each culture was recorded at 5 min. intervals over 40 mins. The mean and standard deviation for the control and treatment groups were calculated at each time interval and graphed. A student T-test assuming unequal variance at P = 0.05 significance level, was performed on the null hypothesis that there was no significant difference between the distance grown over time of untreated *S. ferax* hyphae and GTPγ-S treated *S. ferax* hyphae.

Determining growth recovery time of control and GTPγ-S treated cultures

Twelve *S. ferax* hyphae segments (approx. 0.2 mm in length) were cut aseptically and incubated in sterile watch glasses containing GYPSL overnight at 23°C. Cultures were then randomly selected and equally distributed into either the control or 100 µM GTPγ-S treatment (20 and 40 mins) groups. Each culture was washed once with GYPSL before 200µl of GYPSL or 100µM GTPγ-S was added to the control and treatment groups respectively. The four control cultures were exposed to GYSPL for 40 mins. Four cultures from the treatment group were exposed to 100 µM GTPγ-S for 20 mins. The four remaining treatment cultures were exposed to 100 µM GTPγ-S for 40 mins. After each exposure time, each culture was washed three times with GYPSL before being mounted onto a slide by gently sliding a coverslip under the culture to lift it out of the watch glass. The coverslip was then inverted onto a drop of GYPSL on a slide. Teflon spacers were positioned on either side of the drop of GYPSL or 100 µM GTPγ-S to prevent physical restrictions to the growth of the hypha. The initial position of five randomly selected hyphal tips from each culture was recorded. The selected hyphae were observed every 5 mins. The growth recovery time was defined as the time elongation of the hyphal tip was first observed. A student T-test assuming unequal variance at P = 0.05 significance level, was performed on the null hypotheses that there was no significant difference in recovery times between untreated *S. ferax* hyphae and *S. ferax* hyphae treated with GTPγ-S for 20 mins and between *S. ferax* hyphae treated with GTPγ-S for 20 and 40 mins.

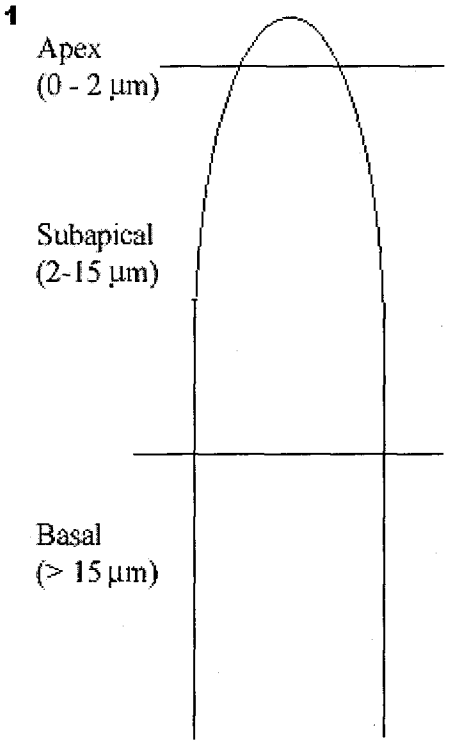


Figure 1. Definition of the apical, subapical and basal regions of *S. ferax* hyphae. There are structural differences in *S. ferax* hyphae proximal to the hyphal tip. Distinction of these regions is important for interpreting changes that may result from GTPγ-S treatment.

Microscopes

The differential interference contrast (DIC) and fluorescent images were taken using a Nikon Eclipse E800 microscope fitted with a HCX PL APO 63×/1.20 W CORR objective and DIC filters, using Sensicam 12bit Cooled Imaging (PCO CCD Imaging), Camware v 1.21 (PCO) software and processed with Adobe Photoshop 7 (Adobe Systems). Vacuole fluorescence was observed using B2A filter (450–490 nm excitation) and super high pressure Hg Lamp (Nikon). Ultrathin sections were observed using the Zeiss902 EM902A and analysed using Digital Micrograph 3.3.1.

Growth measurements were recorded using Olympus CH-2 Compound Microscope fitted with a 40× Olympus objective (E A40, 0.65).

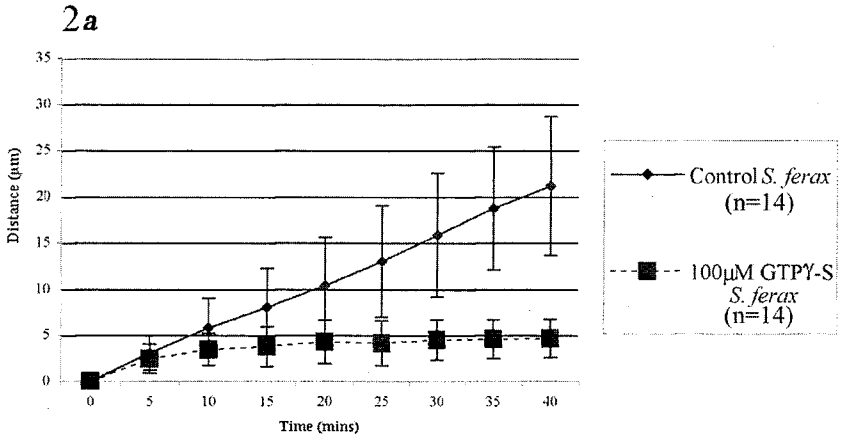


Figure 2a. Tip extension over time by GTPγ-S treated and untreated *S. ferax* hyphae. Growth of untreated or control hyphae (—●—) appeared to be uninhibited over 40 mins. Tip extension of treated hyphae (---■---) appeared to plateau around 35 mins.

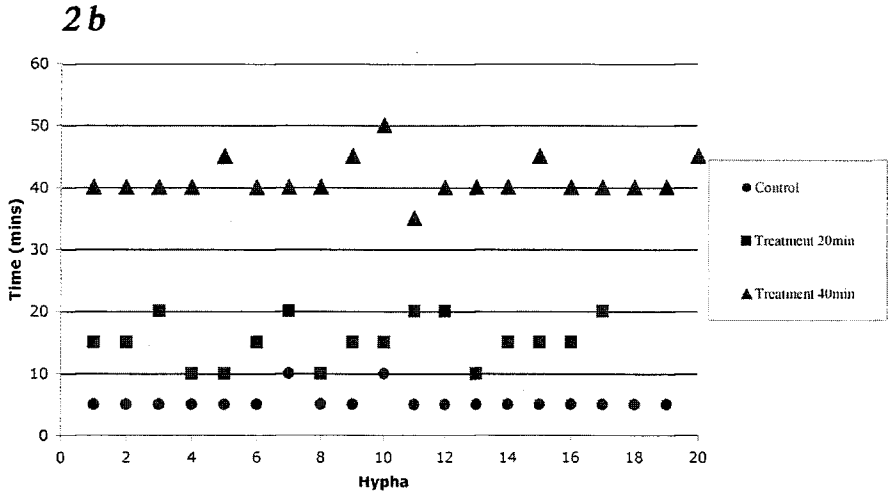


Figure 2b. Individual growth recovery times of GTPγ-S treated and untreated *S. ferax* hyphae. The resumption of growth in untreated hyphae (○), 20 mins (△) and 40 mins (▲) 100 µM GTPγ-S were significantly different. There is a direct correlation between length of exposure to 100 µM GTPγ-S and time elapsed before recovery.

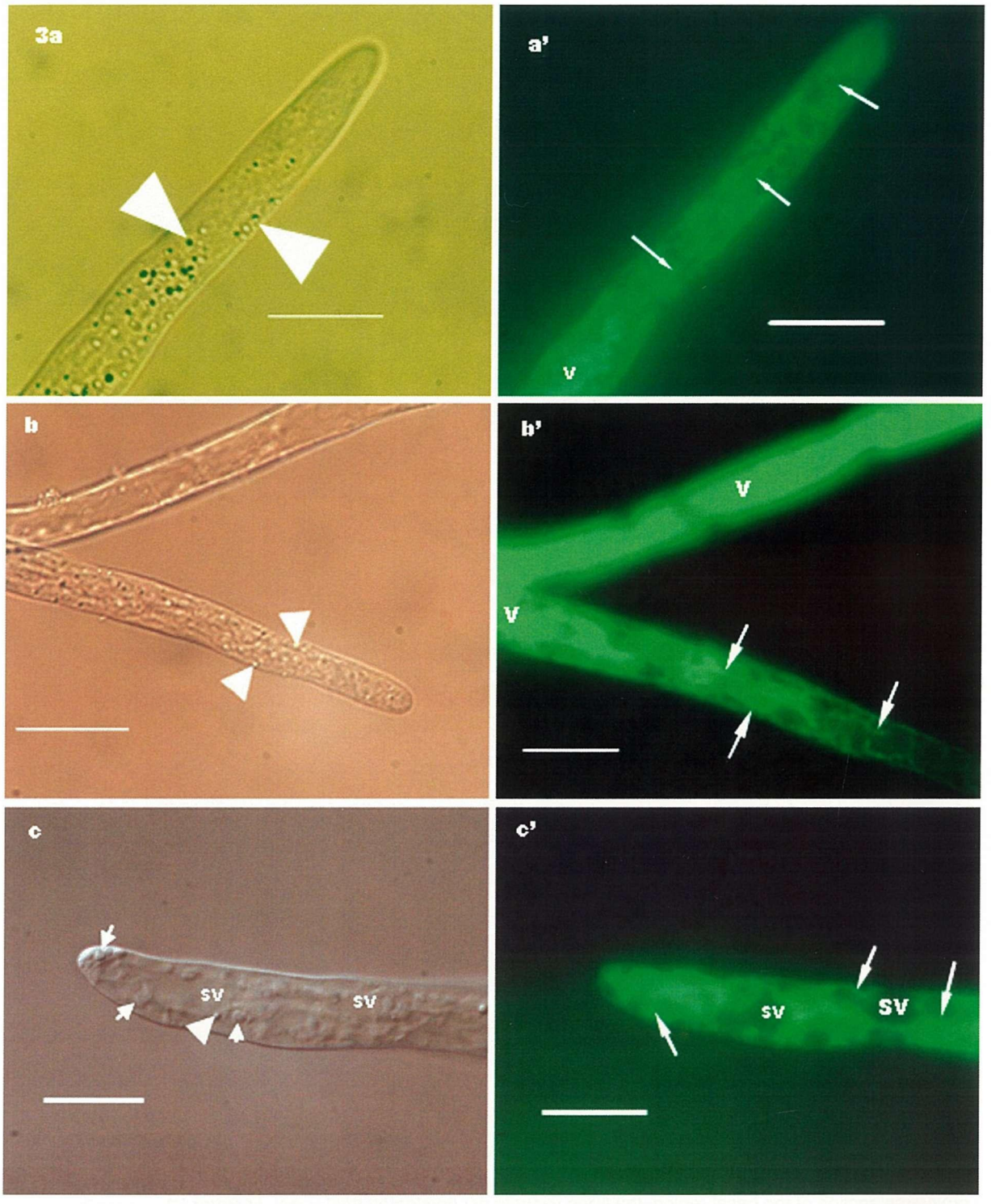
Figure 3. (page 73). DIC and fluorescent images of untreated and GTPγ-S treated hyphae.

In untreated hyphae, the circular organelles (arrowhead) were observed by DIC in the subapical region (approx. 15 µm distal to the apex) of *S. ferax* hyphae (a). The large vacuole (V) was observed around 20–25 µm distal to the tip and the fine reticulate structure branched into the apex of the hyphae (long arrows)(a').

After 20 mins treatment with GTPγ-S, the circular organelles (arrowhead), which may be nuclei, were observed approx. 10 µm distal to the apex (b). The large vacuole (V) was observed further away from the apex. The large vacuole was connected to the tubules of the vacuolar reticulum (long arrows) that extended into the apex (b').

After 40 mins treatment with GTPγ-S, spherical vesicles (SV) and spherical vesicles (arrowheads) were observed in DIC images within 10 µm of the apex. Larger spherical organelles (short arrows) were also prominent within the first 10 µm of the apex (c). The spherical vesicles (SV) interfered with carboxy-DFDDA labelling and the visualisation of the tubules of the vacuolar reticulum (long arrows) (c').

(Bar = 10 µm.)



Results

The fluorescent images in combination with TEM micrographs of *S. ferax* hyphae provided complementary information about the relationship between the fine reticulate structure in the apical and subapical region and the dominant vacuole in the basal region, and how the features of the reticulum and large vacuole change relative to each other with and without treatment with GTPγ-S. In the following description, the apical region was defined to be the area from the apex to 2 µm in from the apex. The sub-apical region was defined as the area 2 to 15 µm from the apex and the basal region 15 µm and beyond (Figure 1).

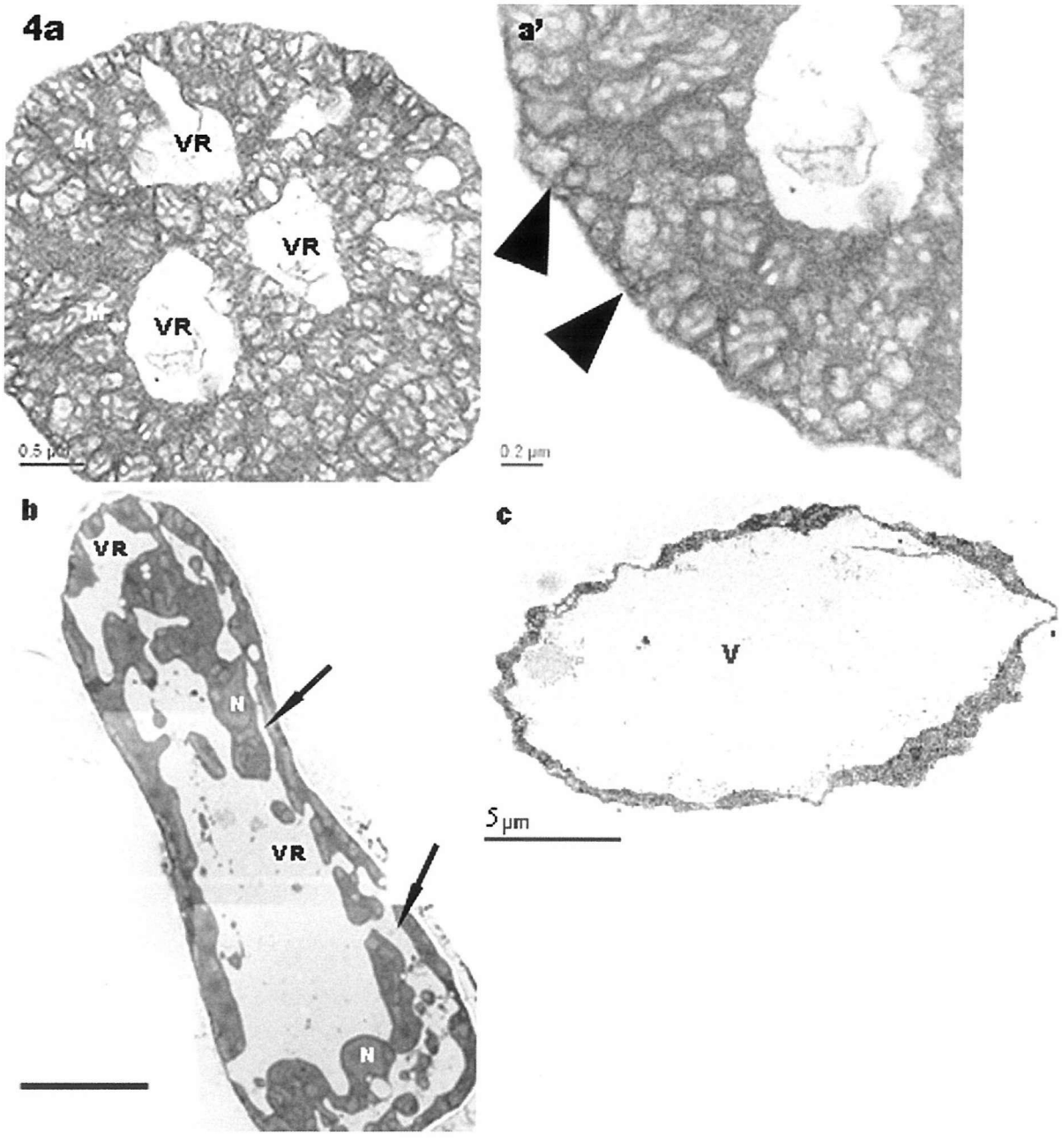


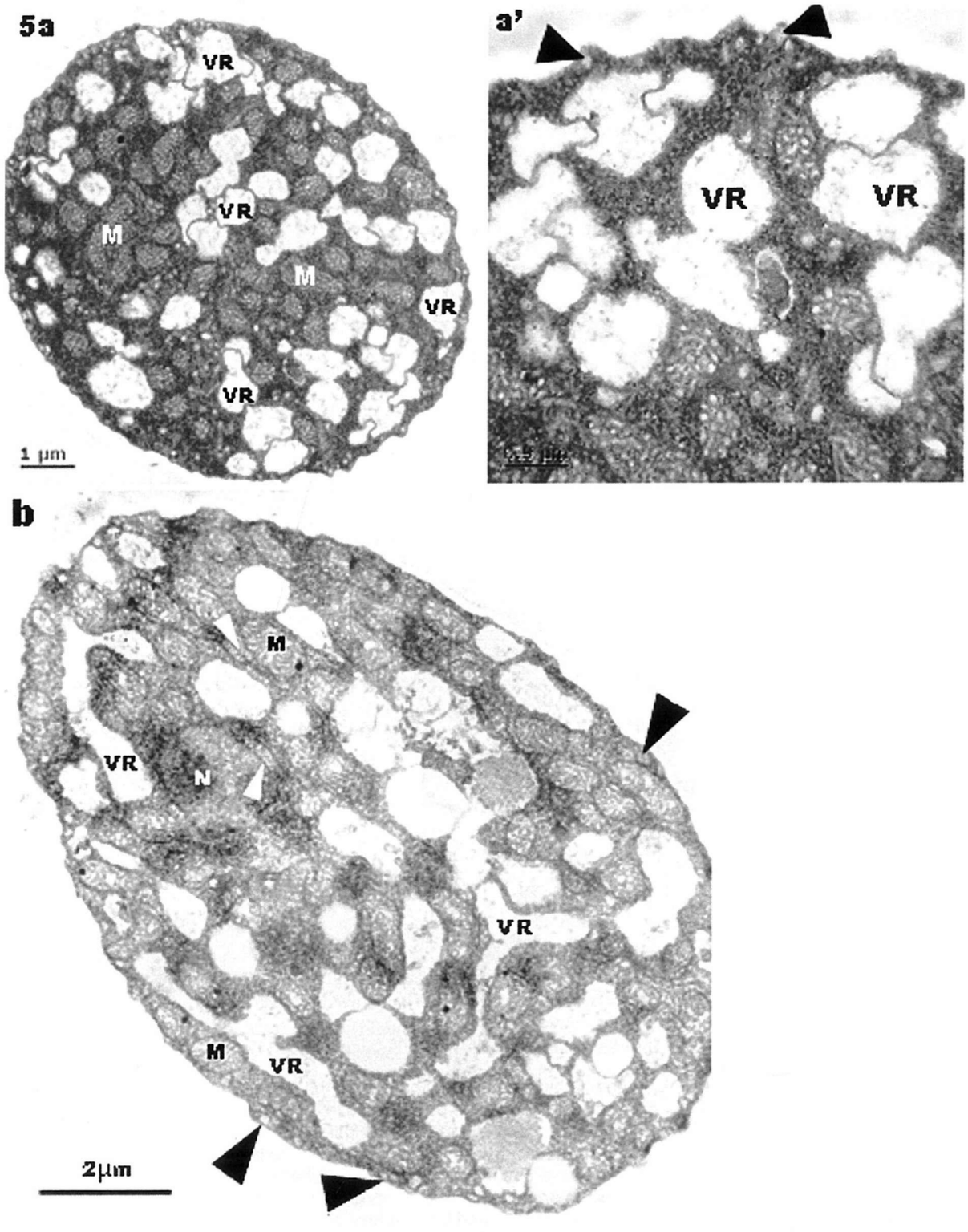
Figure 4. TEM micrographs of untreated *S. ferax* hyphae.

Transverse section of the apical region showing the tubules of the vacuolar reticulum (VR) located towards the centre of the hypha and densely packed mitochondria (M) (a). Small dense vesicles (arrowhead) were observed around the periphery at the apex (a'). Longitudinal section of the subapical region shows interconnection between the tubules (long arrows) of the vacuolar reticulum (VR) with nuclei scattered in amongst the branches (N)(b). Transverse section of the basal region, the large vacuole (V) dominated the hyphal diameter (c).

(Bar = 10 µm, unless otherwise stated.)

Figure 5. (page 75). TEM micrographs of GTPγ-S treated hyphae after 20 mins.

Transverse section of the apical region showing increased number and overlapping of tubules of the vacuolar reticulum (VR). The vacuolar reticulum is located in amongst the mitochondria (M) including the periphery (a). Small dense vesicles (arrowhead) are located around the periphery near the vacuolar reticulum (VR) in the apical



region (a'). Transverse section of the subapical region showing a random distribution of nuclei (N), mitochondria (M), endoplasmic reticula (white arrowhead) and the branching of the vacuolar reticulum (VR). Small dense region (a'). Transverse section of the subapical region showing a random distribution of nuclei (N), mitochondria vesicles (black arrowhead) were also observed around the periphery (b).

(Bar = 10 µm, unless otherwise stated.)

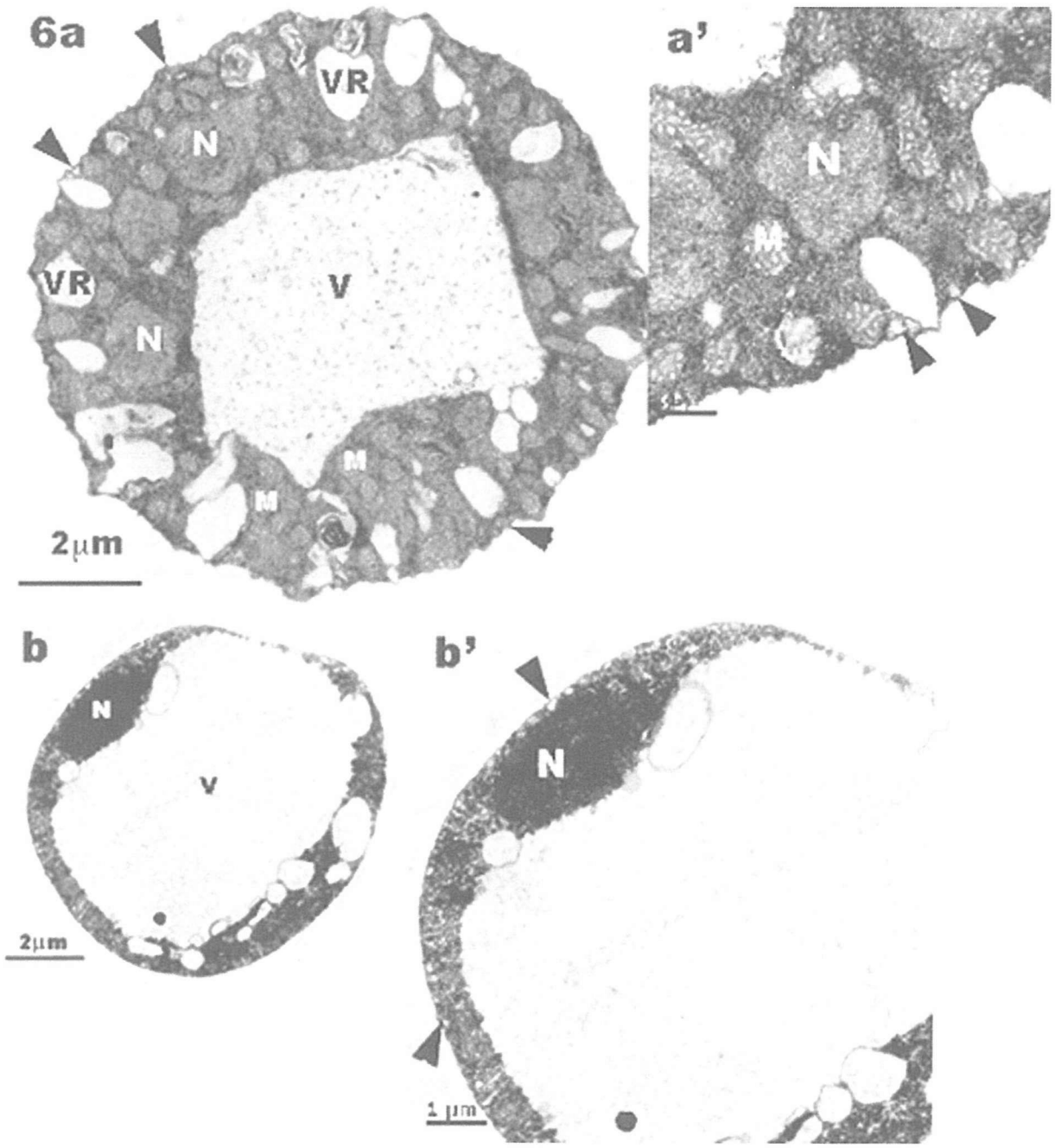


Figure 6. TEM micrographs of GTPγ-S treated hyphae after 40 mins.

Transverse section of the subapical region of a hypha, showing the start of the large vacuole (V) connected to the vacuolar reticulum. Accumulation of metabolites is also more pronounced in the large vacuole. Branches of the vacuolar reticulum are scattered randomly around the periphery in amongst the nuclei (N) and mitochondria (M). Some of the structures thought to be linked with the vacuolar reticulum may be the large spherical vacuoles observed in DIC images. Small dense vesicles are observed around the periphery (arrow head) (a). Small dense vesicles were observed around the periphery in amongst the vacuolar reticulum, nuclei (N) and mitochondria (M) (a'). Transverse section of the basal region shows small and large spherical structures around and within the vacuole (V) in the distal region of the vacuolar reticulum. These may also include extended branches of the vacuolar reticulum. Nuclei (N) are also observed in the basal region (b). Accumulation of luminal content was observed in the vacuole. Small dense vesicles (arrowhead) are observed around the periphery in the basal region of the hypha (b').

Growth rate

Statistical analysis of the distance grown (Figure 2a) showed a significant difference between control or untreated *S. ferax* hyphae and GTPγ-S treated *S. ferax* hyphae over time ($t = 9$, d.f. = 13, $P<<0.05$).

Growth recovery time

The mean recovery time after 20 and 40 mins exposure to 100 μM GTPγ-S was 15 mins (SD = ±4 mins) and 41 mins (SD = ±3 mins) respectively. The mean treatment recovery times were both higher than that of the control mean recovery time (6 mins ± 2 mins) (Figure 2b). There was a significant difference in recovery time between control hyphae and 20 mins GTPγ-S treated *S. ferax* hyphae ($t = -10.0$, d.f. = 21, $P<<0.05$) and between 20 and 40 mins GTPγ-S treated *S. ferax* hyphae ($t = -22.5$, d.f. = 32, $P<<0.05$).

Vacuolar reticulum morphology of untreated *S. ferax* cultures

Clearly defined labelling with carboxy-DFFDA indicated specific labelling of the vacuolar reticulum and confirmed endomembrane integrity. The number and width of the fine tubules decreased towards the apex. DIC images suggest the presence of vesicles and mitochondria in the subapical region (Figure 3a). This was confirmed by the presence of densely packed mitochondria in the apical and subapical regions in TEM micrographs (Figure 4a). The tubules of the reticulum did not appear to be closely associated with the plasma membrane as suggested by the fluorescent images. Small dense vesicular structures were closely associated with the plasma membrane in the apical and subapical regions (Figure 4a').

DIC imaging of the basal region showed organelles and nuclei around the periphery of the hypha. The fine tubules of the vacuolar reticulum increased in diameter and eventually connected with the large vacuole (>20 μm) in the basal region (Figure 3a'). Tubules were sometimes visible between the vacuole and the periphery of the hypha in TEM micrographs (Figure 4b). The vacuole eventually extended the width of the hyphal diameter (Figure 4c).

Changes to the vacuolar reticulum of *S. ferax* cultures treated with GTPγ-S

After 20 mins, DIC and carboxy-DFFDA showed external morphology of treated hyphae was consistent with that of healthy control hyphae. (Figures 3b and 3b'). More tubules were observed in amongst packed mitochondria (Figure 5a). Cross-sections of the apex showed the fine reticulate tubules were around the periphery of the hyphae in comparison to the control hyphae (Figure 4a). The tubules at the apex tended to be thicker than in untreated cultures. Small dense vesicles, closely associated with the plasma membrane, were observed in all regions of the hypha (Figures 5a' and 5b). Increased branching of the reticulum was observed in the subapical region. The junction between the tubules of the reticulum and the large vacuole in the basal region receded further from the apex (Figure 3b'). TEM micrographs indicated increased overlapping of membrane bound compartments, which correlates with the accumulation of tubules and/or vesicles in the subapical and basal region (Figures 5a and 5b).

After 40 mins, branching of the fine reticulate structure increased in the apical and subapical regions. Large vesicular structures lacking fluorescent staining were observed by DIC in the apical and subapical regions (Figures 3c and 3c'). The large vacuoles in the basal region of hyphae became more irregular in shape (Figures 6a and 6b). Specificity of fluorescent labelling of the vacuolar reticulum suggests the integrity of the tonoplast was maintained after treatment. Large spherical vacuoles lacking fluorescent labelling were observed in the subapical and basal region (Figures 3c and 3c'). TEM micrographs indicated an increase in the number of membrane bound compartments in all regions. Small dense vesicles were closely associated to the plasma membrane in all regions (Figures 6a' and 6b').

Discussion

GTPγ-S treated hyphae exhibited increased vesiculation in the apical and subapical regions and increased tubulation in the basal region. This was particularly emphasised after 40 mins incubation with GTPγ-S. Increased branching of the vacuolar reticulum and accumulation of small dense vesicles under the plasma membrane were observed in the apical region. The tubules of the vacuolar reticulum became more laterally located in the apical region in cross-sections of treated hyphae compared with control or untreated hyphae. The presence of large non-fluorescent vesicles in the subapical and basal regions of treated hyphae was absent in untreated hyphae. Large vesicles appeared between the large vacuoles and the plasma membrane of treated hyphae reducing vacuole dominance in the basal region compared with control hyphae. Small dense vesicles were also observed closely associated with the plasma membrane in the basal region. More distinct vesicular structures were observed after 20

mins treatment with GTPγ-S than at 40 mins. The continued production of membrane without deposition can result in the accumulation of endomembrane and ultrastructural distortion. Apical extension in GTPγ-S treated hyphae was significantly reduced over time suggesting reduced membrane deposition despite the accumulation of intracellular membrane. By 40 mins, *S. ferax* cultures exposed to 100 μM GTPγ-S stopped growing whereas untreated *S. ferax* cultures continued to grow. Treatment with 100 μM GTPγ-S for 20 mins and 40 mins was observed to be reversible and correlated directly with the length of incubation. Time elapsed before growth resumption increased significantly between untreated, 20 mins and 40 mins 100 μM GTPγ-S treated *S. ferax* hyphae. The growth delay exhibited by untreated hyphae is believed to be due to the disruption caused by the transfer of the culture from the watch glass to the slide.

The presence and accumulation of vesicles in GTPγ-S treated hyphae suggests fusion of vesicles is inhibited while their formation is unaffected. Monomeric GTPase are molecular switches, 'switching-on' when GTP is bound to GTPase and 'switching-off' when hydrolysation of GTP to GDP occurs with its subsequent release (Alberts *et al.* 2002). GTPγ-S is a non-hydrolysable analogue of GTP which when bound to GTPase prevents the binding protein from 'switching-off' (Gilman 1987). GTPγ-S treatment resulted in increased vesiculation in the apical and subapical region and increased tubulation in the basal region of *S. ferax* hyphae. The structural change to the vacuolar reticulum implies regulation by more than one GTPase protein. Regional differences suggest there is functional specialisation of the vacuolar reticulum as reflected by GTPase activity along the length of *S. ferax* hyphae. Hyde *et al.* (2002) noted that GTPγ-S, brefeldin A and aluminium fluoride had localised effects on the vacuolar system of *Pisolithus tinctorius* and similarly suggested differential regulation of the system along the length of the hypha.

Ultrastructural changes in the presence of GTPγ-S support the suggestion that the vacuolar reticulum is actively involved in the transfer of membrane to the growing tip. The concept of the reticulum being the source of nascent membrane in *S. ferax* has been suggested previously (Bachewich and Heath 1998). The continuity of the vacuolar reticulum from the basal region to the apex allows efficient transfer of luminal content and membrane to the growing hyphal tip. Nascent membrane transport in *S. ferax* is more tubular in nature unlike the transient vacuolar/tubular transport mechanism associated with higher fungi (Cole *et al.* 1998, Mironov *et al.* 1997). The accumulation of vesicles and the increase in branching of the vacuolar reticulum in the presence of GTPγ-S, highlight the dynamic nature of the reticulum. The presence of small dense and large vesicles in the basal region after treatment with GTPγ-S, suggest membrane trafficking may extend into the basal region. It is hypothesised that the vacuolar reticulum acts as a membrane reservoir that sequesters membrane produced by the endoplasmic reticula and modified by the golgi bodies in the subapical and basal regions. The sequestered membrane moves continuously to the apical region as part of the vacuolar reticulum and is released as small dense vesicles in the apex for deposition in the plasma membrane.

It has been well established that *S. ferax*, like filamentous fungi, undergoes apical growth (Garrill and Davies 1994, Gupta and Heath 1997, Heath 1987). The presence of dense vesicles in untreated hyphae and their enhanced expression in GTPgamma-S treated hyphae suggest the vesicles have some role in nascent membrane deposition. It is likely that these structures may be similar to the 'walled vesicles' referred to by Heath (1987). However, dense vesicles were also observed in the basal region of hyphae indicating a heterogenous role of the vesicles in different locations. Small dense vesicles in the basal region may facilitate membrane trafficking from the endoplasmic reticulum and golgi bodies prior to sequestration by the large vacuole.

The large vesicles observed by DIC, interfered with selective fluorescent labelling with carboxy-DFDDA. This implies that carboxy-DFDDA was not being transferred across the large vesicle membrane into the lumen in its anionic form (Cole *et al.* 1997). The structures appear to be similar to the large spherical vacuoles described by (Bachewich and Heath 1999). However, the spherical vacuoles did take up a diacetate probe and fluoresce.

The build-up of metabolites or luminal content along the length of the vacuolar reticulum in treated hyphae suggests GTPase involvement in metabolite transport. Microtubules have been associated with saltatory movement of fluid in *P. tinctorius* (Shepherd *et al.* 1993). It is possible that disruption of microtubule assembly by GTPγ-S in *S. ferax* influences metabolite retention in the reticulum. It would be advisable that further elucidation of GTPase involvement in the cytoskeleton of *S. ferax* be attempted. The diversity and function of GTPase (Alberts *et al.* 2002), implies that changes that

result from perturbation or modulation of GTPase activity would involve complex interactions between organelles, endomembrane systems and the cytoskeleton. This study, however, provides supporting evidence to the idea that the vacuolar reticulum retains similar functional characteristics to that of the vacuole system in filamentous fungi by degrading macromolecules, storing metabolites, maintaining cytosolic ion homeostasis and long distance transport of molecules in hyphae (Hyde and Ashford 1997, Klionsky *et al.* 1990, Shepherd *et al.* 1993).

In conclusion, our results suggest that there is a variety of GTPases involved either directly or indirectly with the activity of the vacuolar reticulum of *S. ferax*. Modulating GTPase resulted in localised changes to the vacuolar reticulum suggesting differential function along the hyphal length. The reticulum appears to facilitate membrane trafficking towards the apex by sequestering membrane in the subapical and basal region and depositing it proximal to the apex.

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