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Induction of contour sensing in *Aspergillus niger* by stress and its relevance to fungal growth mechanics and hyphal tip structure

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Abstract

Thigmotropism (contour sensing) has been assigned an important role in both plant and human fungal pathogens. However, outside these systems, our knowledge of the function of thigmotropism in fungal growth control is relatively poor. Furthermore, the effects of environmental stress on thigmotropic responses have received scant attention. To try to elucidate some of the mechanisms behind hyphal contour sensing in response to nutrient-poor environments, we have used micro-engineered substrates and several imaging techniques to investigate the thigmotropic reactions of the ubiquitous fungus *Aspergillus niger*. This organism not appear to demonstrate thigmotropic growth under normal conditions. Our results show that *A. niger* undergoes significant morphological changes during growth on solid substrates and demonstrate that the intensity of contour sensing varies depending on the area of the hyphal tip which initiates contact with the substrate. We propose that growth under nutrient-limited conditions triggers several factors that combine to increase thigmotropic sensitivity while conversely creating a 60° arc at the hyphal tip which is blind to topographical variations. This has important consequences for our general understanding of the hyphal mode of growth in fungi as well as more specific aspects of hyphal tip development under stress.

Keywords: Thigmotropism; Contact sensing; Hyphal tip; *Aspergillus niger*; Stress

1. Introduction

Hyphal growth of fungi is a fascinating and extraordinarily complex process. It is a defining characteristic of fungi and yet, despite enormous efforts, is still only partially understood. This is mainly due to the multitude of exquisitely controlled interactions that must occur between a variety of different biological building blocks to create a fully functional cell wall. Unlike spherical bacterial cells, which may theoretically extend in any direction, growth of the hypha is extremely polar, with the vast majority of cell wall deposition localized within a few micrometers of the growing tip (Harold, 2002; Bartnicki-García, 2003). In recent years, there have been two main hypotheses regarding the formation of the hyphal apex. The steady-state (SS) model of Sietsma and Wessels (1994) proposed that plastic wall material is continually deposited at the hyphal apex and cross-linked into a more rigid form over time. The inherent turgor pressure within the hypha incessantly drives the semi-fluid tip forward, during which period the rigidifying wall forms a solid base at the foot of the apical dome. The second hypothesis revolves around the concept of a vesicle supply centre (VSC) (Bartnicki-García et al., 1995). This model predicts that the Spitzenkörper, or equivalent structure, acts as a distribution point for vesicles containing cell wall synthesizing materials. It suggests that

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a gradient of exocytosis would be created as this vesicle assembly point moves with the growing hyphal tip. It is this gradient that is hypothesised to be responsible for the shape of the apical dome.

Bartnicki-García (1999) later proposed a model which merged the two theories. This combined model was based on research which demonstrated that vesicles derived from the Spitzenkörper were able to render any cell wall region plastic (Bracker et al., 1997). This ability is thought to be governed by enzymes known as lysins which are located in the vesicles (Money and Hill, 1997; Hill et al., 2002). These wall-softening agents maintain the plasticity of the cell wall for a short period after it has been deposited. Overall however the limiting factor on the size and shape of the fungal hypha is the flexibility of the cell wall. The combined model predicted that this will be enhanced by the fluid nature of the initial cell wall compounds and the action of lytic enzymes on the existing cell walls. Uncontrolled expansion and eventual rupture of the cell wall would be prevented by the rigidification of wall polymers, as predicted by the SS model, and inactivation of the lytic enzymes. Bartnicki-García (1999) concluded that the VSC theory explains the spatial organization of the fungal tip while the SS model accounts for the temporal control of wall flexibility.

The cell wall is supported by a complex network of cytoskeletal components within the hypha which include actin filaments (F-actin) and microtubules. F-actin has been shown to play a key role in tip growth as numerous studies have shown that exposure to anti-actin drugs causes growth defects (Geitmann and Emons, 2000; Heath et al., 2000; Sampson and Heath, 2005). The role of microtubules in tip extension and support is less obvious as growth is able to continue even after microtubule depolymerisation, although growth rate is limited and tip structure is often distorted (Sampson and Heath, 2005; Horio and Oakley, 2005). Overall it appears that F-actin, or its substitutes, perform critical functions in tip extension and stability while microtubules perform less important roles, although the functional details of both systems are the subject of major debate.

Comprehensive information regarding the sensing of environmental influences and their interpretation into directional control mechanisms is also relatively sparse. However several factors have been identified that influence the guidance of the fungal hypha including electrical fields (galvanotropism) (Lever et al., 1994; Gow, 2004), chemical influences (chemotropism) (Fomina et al., 2000; Sbrana and Giovannetti, 2005) and topographical sensing (thigmotropism) (Watts et al., 1998; Apoga et al., 2004). Thigmotropic reactions are thought to play an important role in human and plant pathogenesis and have been studied in rusts, cereal pathogens and *Candida albicans* (Watts et al., 1998; Apoga et al., 2004; Perera et al., 1997; Tucker and Talbot, 2001; Jaffe et al., 2002). Current ideas regarding the control of both fungal growth and thigmotropism centre on stretch-activated calcium channels which are located in the fungal cell membrane and react to its deformation (Watts et al., 1998; Shaw and Hoch, 2000; Silverman-Gavrila and Lew, 2002). However, the positioning and specific mode of action of these Ca^{2+} channels is still subject to debate.

To try to elucidate some of the mechanisms behind hyphal tip expansion and thigmotropic control we have developed a method to quantify contour sensing in complex hyphal systems. Using this technique we have made the novel discovery of thigmotropic behaviour in *Aspergillus niger* under low-nutrient conditions, observed variations in the strength of these interactions based on physical and nutritional parameters and have also investigated the profile of the *A. niger* hyphal tip under nutrient stress. Combining these separate sets of data has allowed us to propose a hypothesis regarding the cause of thigmotropic activity in *A. niger* under low-nutrient conditions and the structural and functional changes that lead to this behaviour.

2. Materials and methods

2.1. Channel slide preparation

Clean quartz microscope slides were spin coated with S1813 photoresist at 25 g for 4 s and then 900 g for 20 s to ensure a uniform thickness. The slides were then placed on a hotplate at 90 °C for 10 min to harden the resist prior to exposure. The samples were then irradiated for 3 s using UV light (365 nm) through a mask patterned with the 20 μm lines. The resist was then developed using CD26 developer for 15 s, washed with deionised water, dried using nitrogen and then post-baked on a hotplate for 20 min at 120 °C. Using the patterned resist as a mask, the slides were placed in buffered HF etch for 20 min. After removal, the remaining resist was stripped using acetone. The slides were then finally washed and dried as before.

2.2. Experimental set-up

Channel slides (Fig. 1A) were placed on top of four other plain microscope slides to allow sufficient elevation for a reasonable depth of agar around them. All slides had previously been washed in detergent overnight, autoclaved and dried in an oven at 100 °C for 24 h to sterilize them and remove any moisture from their surfaces. The stack of slides was then placed in a Petri dish and surrounded with malt-extract agar up to the level of the top slide (Fig. 1B). They were then inoculated with the ubiquitous fungus *A. niger* van Tieghem (ATCC 201373) and incubated at 27 °C for 3 days. Plates were sealed to preserve a humid atmosphere around the colony. Control experiments used an identical set-up, but substituted the channel slide for a blank microscope slide.

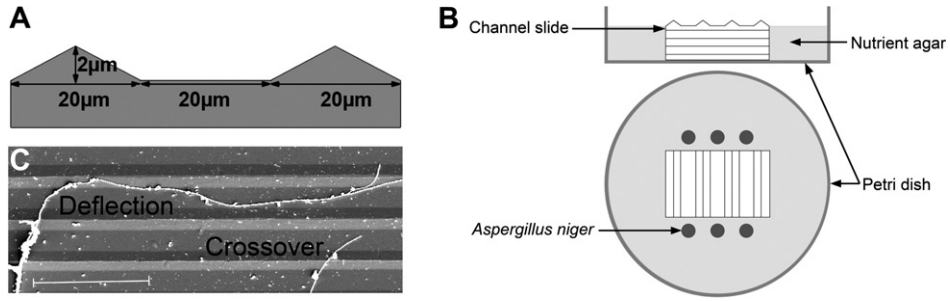

Fig. 1. Channel slide structure and experimental design. The engineered slide had a repeating pattern of 20 μm channels with angled edges etched into a quartz matrix (A). In the final experimental set-up (B) the channel slide was placed in a Petri dish on top of four microscope slides to allow a sufficient depth of agar around it. Nutrient agar was added up to the level of the engineered slide to enable the fungus to easily invade the experimental substrate. *A. niger* was inoculated around the edges of the slide and allowed to grow for 5 days before visualisation. The electron micrograph (C) demonstrates the two parameters used to estimate the levels of thigmotropic behaviour.

2.3. Image analysis

Imaging was carried out using a Leica DM 4000 M microscope and 'Capture' image capture package. 300×300 μm sections on the outer edge of the channel slide were chosen using a numbered grid and random number generator. Images were taken on a direct line inwards up to the tip of the growing mycelia. Pictures taken were analysed using ImageJ image analysis software (available at http://rsb.info.nih.gov/ij/) and a specially designed Excel spreadsheet. The experiment was repeated three times and a total of 11 sections were examined. This represented the analysis of 3504 hypha–ridge interactions.

2.3.1. Data analysis

Images were analysed to determine the level of hyphal reorientation while the hyphae were in contact with the ridges. To establish this only those hyphae that interacted with ridges while reorientating were taken into account. ''Deflections'' were classed as hyphal movements during which the tip entered onto a ridge, did not pass the halfway point and then turned to emerge from the same side of the ridge (Fig. 1C). ''Crossovers'' were defined as hyphae that grew onto and emerged on different sides of a ridge (Fig. 1C). Control samples were grown on flat microscope slides with no ridges. To analyse these we designated 20 μm strips of the images as channels in the same configuration as the physical channels on the manufactured substrates. Hyphal interactions with these superimposed 'channels' were evaluated according to the same criteria for crossovers and deflections as described above.

2.4. Cryo-scanning electron microscopy

Small drops of malt-extract agar were positioned in the centre of glass coverslips cut to 0.25 cm^2. The drops were inoculated with *A. niger* spores and these were allowed to grow overnight in a humid environment until the hyphal tips were protruding onto the glass surface. The glass squares were transferred to a sample holder and plunged into liquid nitrogen under a vacuum in a freezing chamber (Alto2500). The sample was withdrawn from the chamber just prior to the nitrogen solidifying and transferred under vacuum to the preparation chamber. The sample was warmed to −95 °C for 5 min to remove the surface water. After sublimation the sample was cooled to −115 °C prior to coating with approximately 5 nm Au/Pd. Samples were examined using an Hitachi S-4700 field emission gun scanning electron microscope (FEG SEM) operating at an accelerating voltage of 5 kV. To observe hyphal tip morphology under optimal conditions *A. niger* was grown overnight on malt-extract agar plates covered with semi-permeable membranes to act as a control. Small sections of membrane with hyphal tips attached were removed from the plate, mounted on glass and examined as described above.

3. Results

3.1. Variation in thigmotropic response with angle of interaction

Two distinct features of the thigmotropic behaviour of *A. niger* were observed. The first was the apparent dependence of hyphal reorientation on the angle of interaction with the channel wall. Fig. 2A shows the percentage of hyphae that shifted their growth on contact with a ridge as a function of the angle of that interaction. These data show that at angles of approach of less than 20°, over 80% of hyphae responded thigmotropically and altered their growth away from the obstacle. However at angles greater than 20° the level of response fell dramatically, until at 60° and above the response essentially fell to zero.

3.2. Variation in thigmotropic response with colony age/ nutrient availability

The second major feature was the variation in contour responses over distance. Fig. 2B shows the percentage of hyphae that reoriented their growth on contact with a channel wall as a function of the distance from the slide edge (nutrient source). The data shows that the level of thigmotropic response increased as the fungal colony pene-

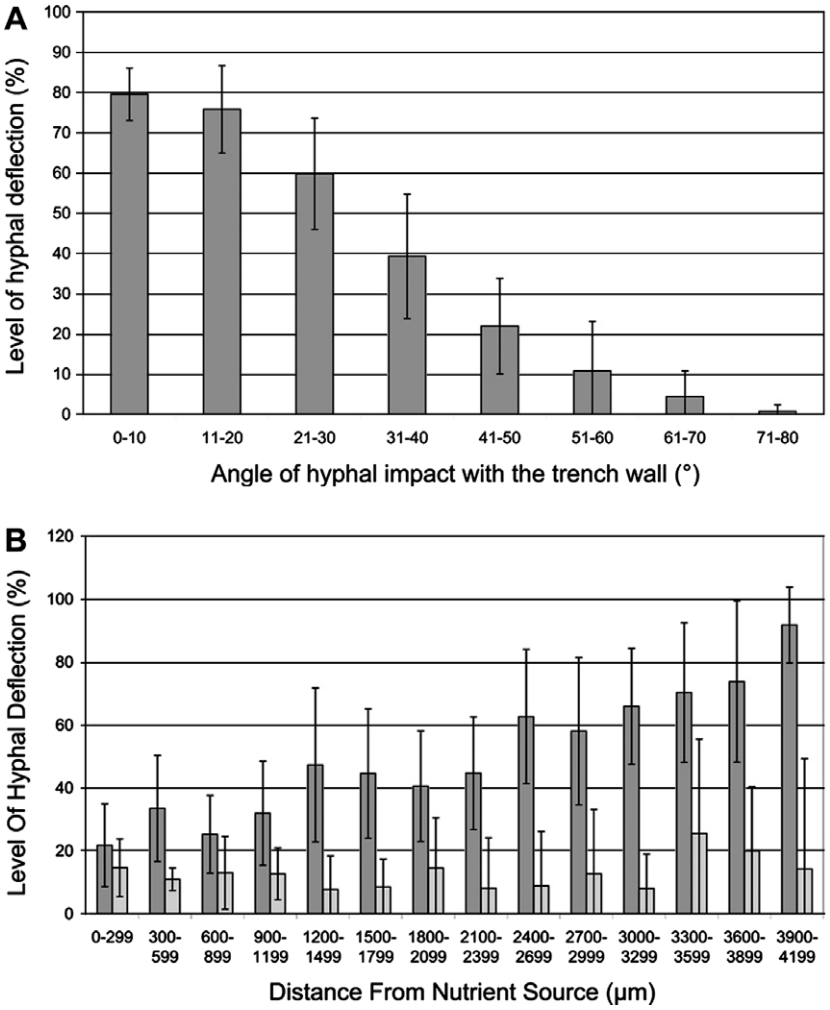

Fig. 2. Variation in thigmotropic response. The strength of the reorientation response also varied depending on the angle of hyphal contact with the ridges (A). At angles of interaction greater than 60° the response was essentially zero, but as the contact angle decreased the levels of reorientation increased to over 80% at angles less than 10°. We also observed that as the fungus penetrated onto the channel slide the levels of thigmotropic response increased from approximately 10% to almost 100% over a distance of 4.2 mm (dark grey bars) (B). Control experiments on slides without ridges (light grey bars) showed that the levels of thigmotropism demonstrated on the ridged substrate could not be random changes in direction.

trated further onto the channel slide and away from the nutrient source. This suggested that the intensity of the contact response varied according to biological or chemical parameters.

3.3. Observation of *A. niger* hyphal tip morphology

To try to better understand why variations in thigmotropic response occur and make predictions on the specific mechanisms underpinning such variations, a more detailed investigation of the morphology of the *A. niger* hyphal tip under stress was required. We initially attempted both light and confocal microscopy of the hyphal tips, but neither provided sufficient resolution to discern the structure of hyphal tips growing on glass. The tips were either too small, or under too much stress to tolerate live imaging. To allow us to capture the detailed images we needed, we inoculated the test fungus onto glass squares (0.25 cm^2) which were examined from a variety of angles using cryo-scanning electron microscopy (cryo-SEM). The main advantage of cryo-SEM is that it almost instantaneously freezes the fungal biomass and requires no processing other than a thin coating of gold-palladium which should preserve the exact shape of the hypha. This allowed us to thoroughly investigate hyphal morphology and its association with the variations we observed in topographical sensing. An examination of our stressed samples grown under low-nutrient conditions on glass (Figs. 3 and 4) revealed striking differences to cultures grown on softer substrates and more amenable nutrient conditions (Fig. 5). Stressed hyphae were much smaller and appeared deflated with an apparently healthy central region surrounded by a lip of slack hyphal material (Fig. 3). The level of deflation varied throughout the samples from severe (Fig. 3A) to minimal (Fig. 3B) and did not appear to correlate with distance from the nutrient source. It was also a characteristic which was not limited to the hyphal tip region as some hyphae demonstrated this stressed morphology behind the hyphal

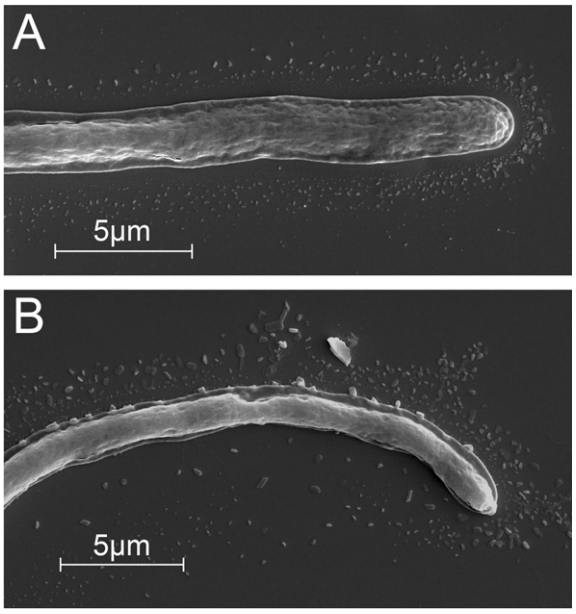

Fig. 3. Analysis of stressed hyphal tips. Using cryo-SEM analysis techniques we were able to observe the structure of the hyphal tips from the top. It appeared that the hyphae had flattened edges around the main body of the hypha (A and B). These compressed areas varied in severity from relatively small (A) to large (B), although there appeared to be no obvious correlation between this characteristic and distance from the nutrient source. There were also obvious haloes of crystals around the hyphae which is indirect evidence that growth and metabolism was occurring up until the point of freezing.

tips (Fig. 3B). One of the main drawbacks to the cryo-SEM technique is that no live-cell imaging is possible. However, the observation of a ring of apparently biogenic crystals surrounding the hypha, coupled with no evidence of hyphal rupture suggest that the hyphae were still viable up to the point of freezing. Visualisation of the profile of hyphae grown under stressed conditions (Fig. 4A–E) supported our observation of deflated morphology in hyphae viewed from the top. When seen from the side we were able to describe two abnormal tip profiles which were present in an approximately 50:50 ratio (data not shown) in samples grown under conditions of nutrient stress. The first appeared almost as a water droplet with a relatively gently sloping wall down to an apex ~0.2 μm in height (Fig. 4B and C) while the second profile we observed had a much more steeply sloped tip which again culminated in an apex ~0.2 μm in height (Fig. 4D and E). It seems likely that in both cases these 0.2 μm high structures correspond to the ridge of deflated cell wall material that surrounds the stressed hyphae. These stressed morphologies are significantly different from the gently rounded tip shape which *A. niger* displays under optimal conditions (Fig. 5A–C). When grown on softer substrates and with easy access to nutrients, the hyphae are approximately double the diameter of those grown under conditions of nutrient stress. They appear fully inflated and exhibit a gently rounded, bulging morphology which lifts the hyphal apex slightly off the ground (Fig. 5B and C).

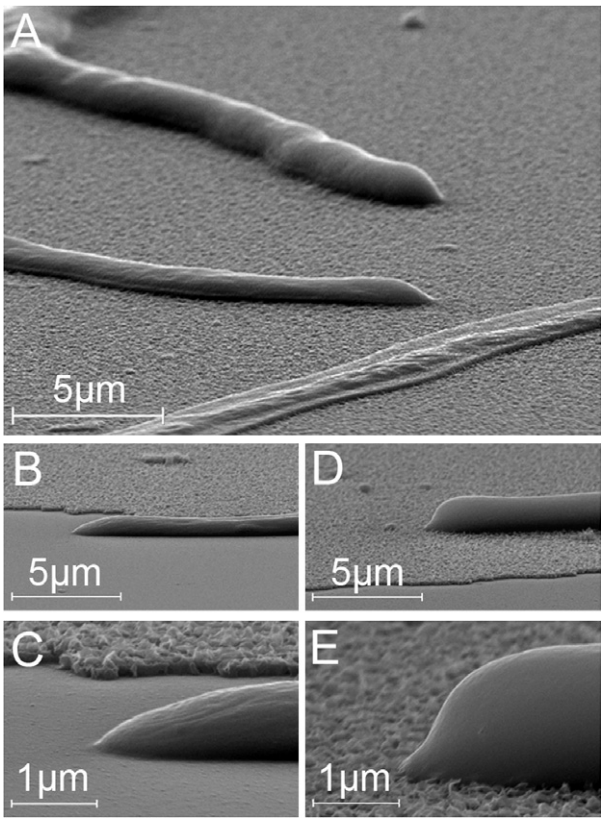

Fig. 4. Hyphal tip profiles of stressed hyphae. Cryo-SEM techniques were used to analyse the detailed profile of *A. niger* hyphal tips during growth on glass. Two main structures were observed, both with significantly smaller profiles at the extreme tip than the main hyphal body. (A) shows an overview of both these major shapes. The hyphal tip in the foreground displays a water droplet shaped profile (shown in more detail in (B) and (C)), while the profile in the background displays a much more distinct microtip (enlarged in (D) and (E)).

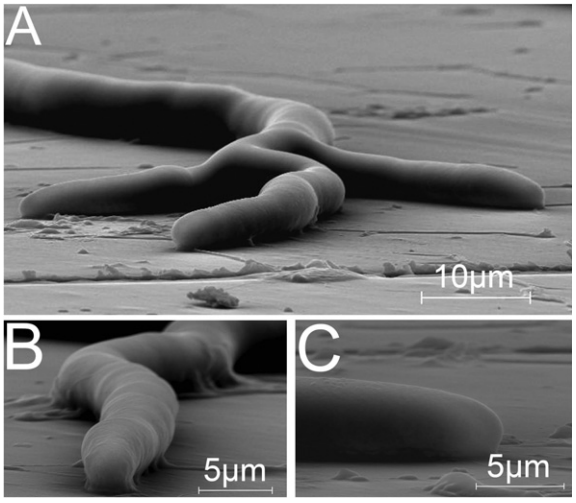

Fig. 5. Hyphal tip morphology under optimal conditions. Cryo-SEM of *A. niger* hyphal tips growing under optimal nutrient conditions on a semi-permeable membrane (A). Fig. 4B shows the tip head demonstrating an almost perfect tubular aspect with only slight flattening towards the apex. The profile (C) perfectly demonstrates the gently rounded spherical tip shape that is normal under non-stressed conditions.

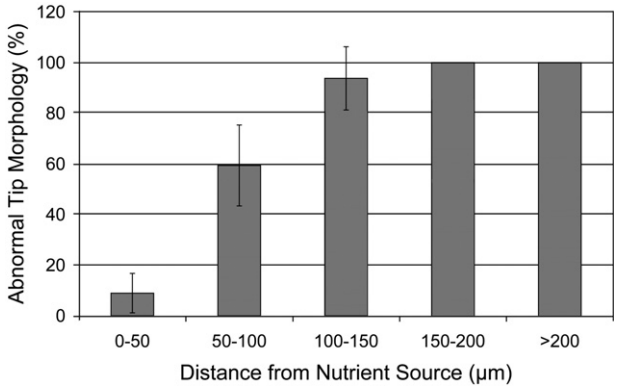

Fig. 6. Variation in stress-related morphological changes. Changes in hyphal tip morphology were found to correlate with the distance of the tip from the nutrient source. Flattening of the tip and other stress-related morphological changes were found to increase in abundance as the hyphae penetrated onto the nutrient-free substrate.

3.4. Tip morphology changes

Using the cryo-SEM we were also able to observe morphological changes as a function of distance from the nutrient source. Our results showed that the appearance of stressed hyphal tip shapes correlate to increasing distance from the agar drops (Fig. 6). At distances of less than 50 µm from the nutrient source more than 90% of the tips displayed the normal, gently rounded apexes typical of *A. niger* under optimal conditions. This level decreased rapidly until at distances greater than 150 µm from the nutrients there was no evidence of normal tip structure and all tips displayed the characteristic flattened profiles described earlier.

4. Discussion

In this research, we have investigated contact sensing responses of the ubiquitous soil fungus *A. niger*. Our data clearly demonstrate that thigmotropic reactions are initiated in response to stress and appear to vary during growth under conditions of nutrient stress. We observed a decrease in the levels of thigmotropism as the angle of hyphal interaction with the channel wall increased (Fig. 2A). These results support those of Watts et al. (1998) in their experiments with *C. albicans* germ-tubes grown on grooved polystyrene membranes which showed that as the angle of interaction increased, the levels of hyphal reorientation fell. These authors also demonstrated a link between these thigmotropic reactions and stretch-activated ion channels by using specific channel blockers. Although the morphogenetic differences between this yeast and *A. niger*, and the experimental systems employed, mean that direct comparisons cannot be made, the fact that very similar results have been obtained suggests that these data may represent a general aspect of hyphal growth. The variation in thigmotropic response to different angles of interaction suggests that there must be a relationship between the position of ridge contact with the hyphal tip and the level of contour sensing. As the angle of approach increases, the point of contact will move further towards the hyphal apex. Thus at an approach angle of 90°, the site of interaction will be at its nearest point to the tip, while as the angle approaches zero the contact will move posterior to the apex. The current hypothesis is that thigmotropic reactions are mediated by stretch-activated ion channels which are present in the cell membrane at the hyphal tip (Perera et al., 1997; Silverman-Gavrila and Lew, 2002). These calcium channels are thought to be connected to the cell wall through the cell membrane via integrin linkages (Kaminskyj and Heath, 1995) and Hechtian strands (Bachewich and Heath, 1997). This plasma membrane-cell wall adhesion system has been widely studied and accepted in plants (Jaffe et al., 2002) but has only recently been explored in fungi. It was found that the cell membrane-cell wall adhesion patterns varied depending on the region of the hypha which was studied. At the tip the connections were broad and irregular, while during the transition zone they displayed a regular and continuous distribution. This in turn changed to an infrequent and punctate set of connections in mature regions of the hypha. We have shown that hyphal tips appear unable to respond to topographical variations at angles of approach greater than 60°. We suggest that this arc at the extreme apex of the hyphal tip is essentially insensitive to obstacles due to a combination of the low density of plasma membrane-cell wall connections combined with the immature nature of both the cell wall and cell membrane (Fig. 7). According to the VSC-SS model (Bartnicki-García, 1999), the cell wall is in a semi-fluid state at the very tip of the hypha and solidifies further back from the apex. This suggests that the extreme apex would be unable to efficiently convey stretch responses due to its fluid, embryonic nature. This prediction has been confirmed by Ma et al. (2005) using atomic force microscopy, which demonstrated that rigidity increased for a distance of 3 µm behind the hyphal tip before stabilizing. Equally the cell membrane is in a state of flux at the extreme apex as vesicles from the Spitzenkörper carry new components to the apical area where they are incorporated into the

Fig. 7. Immature cell wall and membrane at the tip creates a thigmotropic 'Blind Spot'. Our results indicate that stressed hyphal tips are insensitive to topological variations over a 60° arc (grey shading) at the apex (when viewed from above). We propose that this is because the newly deposited cell wall (▪▪▪▪▪▪) is too immature, both in its lack of connections to the cell membrane and physical fluidity, to transmit thigmotropic cues. The plasma membrane ▬ ▬ too will be in a state of flux near the apex as vesicles from the Spitzenkörper constantly introduce new components into the membrane.

expanding membrane. The calcium channels which will be inserted into the plasma membrane during its maturation may not be present, active or attached to the cell wall at this early stage in membrane development.

We also observed that the sensitivity of contour sensing increased as the hyphae moved away from the nutrient source onto the nutrient-free experimental substrate (Fig. 2B). We hypothesise that these fluctuations might be a purely structural response to the increasing levels of stress that the colony will be experiencing over the course of its growth on the channel slide as we have shown an increase in abnormal tip morphology as the hyphae penetrated further into the nutrient free domain (Fig. 6). The cell wall response to chemical stress in *A. niger* has already been examined (Ram, 2004) and it was found that the fungus responded by increasing the levels of chitin in the cell wall to maintain the integrity of the hyphae. If a similar response is activated in response to low-nutrient stress, it is possible that increased levels of chitin might solidify the growing tip more quickly, amplifying its sensitivity to- and conductivity of topographical signals. This might also explain the morphologies we observed with the cryo-SEM on glass which are not typical of *A. niger* under non-stressed conditions as the 'foot' observed around the bottom of the hyphae could be made up of this excess wall material. Previous work on *Puccinia graminis* has demonstrated that during germ-tube growth the lower region of their tip becomes flattened (Read et al., 1992). This morphology is thought to allow close contact between the substrate and the topography-sensing mechanisms in the tip, making it essential for efficient thigmotropism. Our results suggest that stress initiates similar morphological changes in *A. niger* although we can only speculate as to the specific changes in cell wall or cytoskeletal structure that cause this effect. This appears to lead to the development of thigmotropic activity in an organism which does not exhibit this behaviour under non-stressed conditions. We hypothesise that the flattened tip profile observed under stressed conditions would mean that the point of contact with any topological feature would be further forward in the tip in comparison to non-stressed morphology (Fig. 8). This would allow greater time for Spitzenkörper reorientation and reaction to obstacles. In comparison the normal hyphal morphology has a raised apex and no evidence of flattening. This would mean that much of the initial contact with an obstacle would take place behind the Spitzenkörper and that the impact would only affect a small area of the hyphal body. This might induce a calcium signalling response, but it might not be sufficiently polarized to induce a reorientation of hyphal growth. The flattened area we observe around the base of the hypha would also act to amplify the strength of the contact from an obstacle. This 'foot' would act as a lever to increase the strength of the stretch response transmitted via the cell wall-cell membrane connections (Fig. 9). It would also increase the surface area over which cell wall deformation would occur on contact with an obstacle compared to hyphae displaying normal morphology. A combination of these effects would multiply the sensitivity of the stressed hypha to topographical features.

Our research has shown that stress induces morphological characteristics *A. niger* similar to those found in thigmotropic rust fungi. We hypothesise that it is this structural variation which causes the induction of thigmotropic growth in this normal non-thigmotropic fungus. We have found that contour sensing in *A. niger* acts to direct its growth away from increasing gradients, a behaviour also observed in *C. albicans* (Watts et al., 1998). However, the rust fungi have been shown to direct their growth at 90° to ridged structures. This diversity in thigmotropic response suggests that during the course of evolution different species have modified their topographical responses to suit their environments. Rust fungi utilize theirs to find stoma and gain access to the plant, while *A. niger* acts under stress to direct its growth towards channels, which are the most likely location of water and nutrients. We believe that our conclusions provide an important step forward in the understanding the role of thigmotropism in fungal growth and hope that

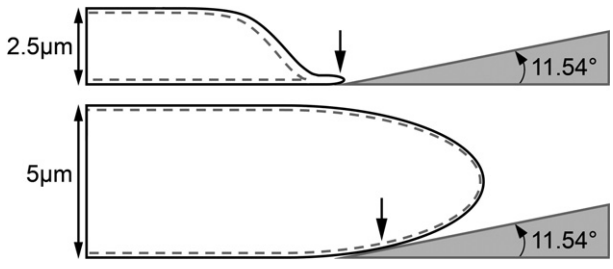

Fig. 8. Advantage of flattened morphology in topographical sensing. The flattened morphology observed in hyphal tips under stress means that the contact point between the hypha and any obstacle would be much further forward on the hyphal body in comparison to a non-stressed hypha. This would mean the majority of any topographical contact would occur at points apical to the Spitzenkörper, allowing the hypha a greater chance of immediately reacting and reorienting itself.

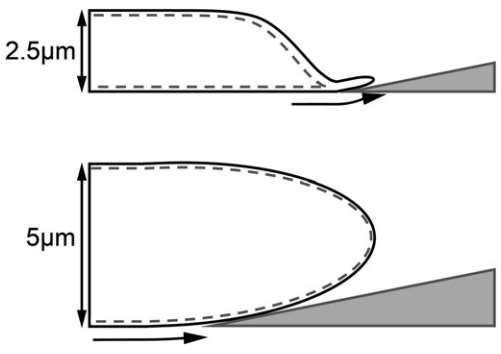

Fig. 9. Flattened morphology amplifies topographical signals. The ridged morphology displayed by nutrient deficient hyphae would serve to increase the stress on the cell wall by acting as a lever as the hypha moves onto an obstacle. The flattened 'foot' of the hypha would also increase the surface area over which deformation of the cell wall would be felt. By comparison a non-stressed hypha would mount an obstacle with relatively little deformation over a smaller surface area.

the theories presented here will serve to catalyze more innovative research into this fascinating, and yet relatively unexplored, area of biology.

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