Protoplasma (2003) 222: 53–59
DOI 10.1007/s00709-003-0006-6

PROTOPLASMA
Printed in Austria

Confocal microscopy of Spitzenkörper dynamics during growth and differentiation of rust fungi

J. Dijksterhuis*

Fungal Cell Biology Group, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh and Centraalbureau voor Schimmelcultures, Utrecht

Received October 25, 2002; accepted February 26, 2003; published online August 26, 2003

Summary. The membrane-selective fluorescent dye FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridium dibromide, was used to stain the apical vesicle cluster within the specialized Spitzenkörper of the germ tube of the rust fungi *Uromyces vignae* and *Puccinia graminis* f. sp. *tritici* grown on glass surfaces. The Spitzenkörper stained within 15 min following addition of the dye. Optical sectioning by confocal microscopy of stained hyphal tips showed that the Spitzenkörper was asymmetrically positioned close to the cell–substratum interface during germ tube growth. The Spitzenkörper showed variations in shape and positioning over short (5 s) time intervals. The movement to a new location in the hyphal dome was followed by new growth in that region, consistent with the view that the Spitzenkörper supplies secretory vesicles for germ tube growth. A pronounced Spitzenkörper disappeared at the onset of appressorium differentiation during swelling of the germ tube. However, a stained structure, similar in appearance to a Spitzenkörper, was again observed during the formation of the highly polarized penetration peg.

Keywords: *Uromyces vignae*; *Puccinia graminis*; Spitzenkörper; Tip growth; Appressorium; Differentiation.

Introduction

Tip growth involves the polarized extension of a walled cell in which the increase in cell length occurs in a region consisting of the hyphal apex and a few micrometers of hypha behind it (Heath 1990). Inside the hyphal apex a characteristic structure, the so-called Spitzenkörper (Spk), is observed by light microscopy of living cells (Girbardt 1957) and electron microscopy (Girbardt 1969, Grove and Bracker 1970, Howard and Aist 1979). It is regarded as an area of strongly localized exocytosis and as the basic organization center of polarized growth of higher filamentous fungi. Video-enhanced optical microscopy of growing fungal apices (López-Franco et al. 1994, López-Franco and Bracker 1996) clearly shows that the machinery responsible for polarized growth in filamentous fungi has a highly dynamic nature.

The germ tubes of many plant pathogenic fungi (e.g., rusts) are highly adapted to efficient infection of host plants. Electron microscopy has shown that rust germ tubes possess an apical vesicle cluster inside the hyphal tip. Most authors have avoided describing this structure as an Spk (Hoch and Staples 1983). Recently Hoffmann and Mendgen (1998) were able to identify with phase-contrast optics a "diffuse dark cloud" within the growing germ tube tip of *Uromyces fabae*.

The fluorescent styryl dye FM4-64 stains the plasma membrane and cannot enter the cell except by endocytosis (Betz et al. 1996, Vida and Emr 1995). FM4-64 has been found to stain the vesicles within the Spk of numerous fungal species (Fischer-Parton et al. 2000, Wedlich-Söldner et al. 2000, Read and Hickey 2001). Germ tubes of the rusts *U. fabae* (Hoffmann and Mendgen 1998) and *Puccinia graminis* f. sp. *tritici* (Fischer-Parton et al. 2000) also show staining of the Spk.

A rust germ tube exhibits remarkable touch-sensing capabilities. This thigmotropism regulates the directional growth of germ tubes and induces their development into appressoria in response to topographical signals on the host surface (Wynn 1976, Hoch et al. 1987, Read et al. 1992). It has been suggested that the close association of the Spk in rust germ tubes with the substrate may be important in the germ tube's ability to sense and respond to surface signals from the underlying substratum (Kwon et al. 1991).

* Correspondence and reprints: Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands.

In the present study we have used confocal microscopy to image living germ tubes of the rust fungi *U. vignae* and *P. graminis tritici* stained with FM4-64. There were three aims to this work. The first was to determine the three-dimensional location of the Spk in growing germ tubes and analyze their dynamic behavior. The second aim was to analyze what happens to the Spk during appressorium formation when the germ tube shifts from the highly polarized growth pattern to the less polarized growth of the swollen appressorium. The third aim was to determine what happens to the Spk when pronounced polarized growth is resumed during the formation of a penetration peg from the appressorium.

Material and methods

Fungal strains, inoculation, and growth substrata

The wheat stem rust, *Puccinia graminis* Pers. f. sp. *tritici* Erics. & Henn. (race 32), and the cowpea rust, *Uromyces vignae* Barc., were kindly provided by B. M. Moerschbacher (University of Münster, Federal Republic of Germany) and M. C. Heath (University of Toronto, Canada), respectively. Urediospores of *P. graminis* (from uredia on wheat) and *U. vignae* (from uredia on cowpea leaves) were inoculated onto flat or microfabricated glass coverslips (nr. 1.5, 22 by 50 mm; Chance Proper, Warley, U.K.) with a painter's brush. To investigate the differentiation of appressoria, glass coverslips with 0.45–0.55 μm high steps were fabricated. Therefore, glass coverslips were cleaned with an oxygen plasma and silicon dioxide was deposited as 0.4 to 0.6 μm (within 90% limits) thick steps. Developing urediospore germ tubes grown on appropriate substrata were kept at room temperature in a humid chamber (consisting of a petri dish with moist filter paper).

Germ tube staining

Staining was done by carefully placing 50 μl of aerated 20 mM HEPES buffer (pH 7.2) containing 3.2 or 6.4 μM N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridium dibromide (FM4-64; Molecular Probes, Eugene, Oreg., U.S.A.) over germ tubes on a coverslip on the stage of an inverted microscope. Germ tubes which were appressed to the glass surface could be observed in this way without further manipulation. A humid environment was created around the coverslip during confocal microscopy by means of an inverted petri dish containing a moist Whatman No. 1 filter paper with a central 1–2 cm diameter hole to facilitate imaging with the conventional transmitted-light optics of the microscope.

Confocal microscopy

Stained germ tubes were imaged with a ×60 plan apo (numerical aperture, 1.4) oil immersion objective in a Bio-Rad MRC 600 confocal system mounted on a Nikon Diaphot TMD inverted microscope (all supplied by Bio-Rad, Hemel Hempstead, U.K.). Dye excitation was obtained with the 514 nm line of an argon ion laser; 1% laser intensity and the GHS filter block (excitation, 514/10 nm; dichroic mirror, 540 nm [long pass]; emission, 550 nm [long pass]) were used. Rust germ tubes were routinely imaged by fast laser scanning (F4 setting, which takes 0.25 s to scan a full-sized image) to localize the position of the Spk. This was done to prevent perturbation of the cell due to the intensity of the laser beam or to phototoxic effects of the dye. Then the final image was acquired with the slower F2 scanning speed (that takes 1 s for a frame). For acquisition of the images at higher resolution with an improved signal-to-noise ratio, the slower F2 scanning setting was used. In addition, Z-series of living germ tubes were made in the F4 mode.

Results

Staining and location of the Spitzenkörper

The germ tubes of *U. vignae* were slightly broader (6.4 ± 0.7 μm, n = 10) than those of *P. graminis* (5.5 ± 0.5 μm, n = 10). Urediospores of *U. vignae* formed only a single germ tube. In contrast, urediospores of *P. graminis* often formed several germ tubes. Only one of these tubes grew over a relatively long distance, while the others seemed to be aborted (data not shown). The growth rates of unperturbed germ tubes on the microscope stage were typically about 1 μm/min. After the immersion of germ tubes in FM4-64 for 10–15 min, the vesicle cloud within the Spk was stained.

The Spk of rust germ tubes were more sensitive to laser irradiation than were the hyphae of most other fungal species that have been studied (see, e.g., Fischer-Parton et al. 2000). Sometimes germ tubes reacted to intensive irradiation by the disappearance of the stained Spk. To prevent this, a quick focusing on the Spk in the fast scanning (F4) mode was done. This procedure took a small number of consecutive frames in the continuous-scanning mode and was followed by the acquisition of the image in one single frame. The Spk was invariably positioned asymmetrically in the germ tube tip close to the cell–substratum interface of both rust species (Fig. 1).

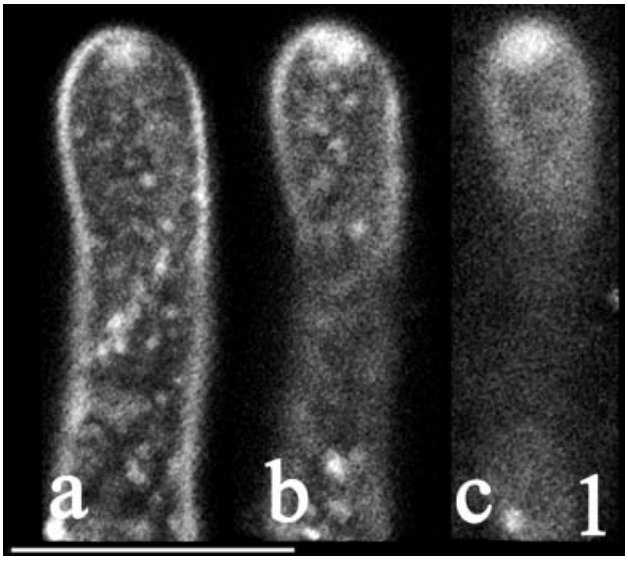

Fig. 1a–c. *Puccinia graminis*. Confocal microscopy of a growing germ tube stained with FM4-64. Optical sections through the germ tube tip show the asymmetric location of the Spk close to the substratum surface. The sections closest to the glass surface (**c** and, to a lesser extent, **b**) reveal intense staining of the Spk compared with that seen in a near median section (**a**). Bar: 10 μm

This was confirmed when Z-series were made of germ tubes of both species. The combined observations, with single scans and Z-series made it clear that the vesicle cloud did not disappear during the acquisition of the Z-series, which in such a way could introduce the "asymmetric positioning" of the Spk. Focusing on germ tubes that had no Spk resulted in a medium section, while the clear Spk when present could quickly be localized near the substratum.

Figure 2 shows the results of a 1 h time course study on the staining of a *P. graminis* germ tube. The pattern of staining was similar for both rust species. Following the addition of FM4-64, the plasma membrane stained immediately. After 2–3 min, cytoplasmic inclusions (0.6–0.8 μm in diameter) began to stain and were readily discernible after about 10 min. After about 15 min, larger stained inclusions became visible within the cytoplasm (Fig. 2). Occasionally, strongly stained spots were associated with the plasma membrane (data not shown). As indicated earlier, the apical vesicle cloud within the Spk became fluorescent between 10 and 15 min and typically appeared as a relatively diffusely stained vesicle structure rather than as an intensely stained region (Figs. 1–3). The size of the vesicle cloud was measured in different images. Spk of *U. vignae* were slightly larger (2.2 by 1.7 μm, n = 7) than those of *P. graminis* (1.7 by 1.3 μm, n = 7). This was consistent with the difference in diameter of the germ tubes.

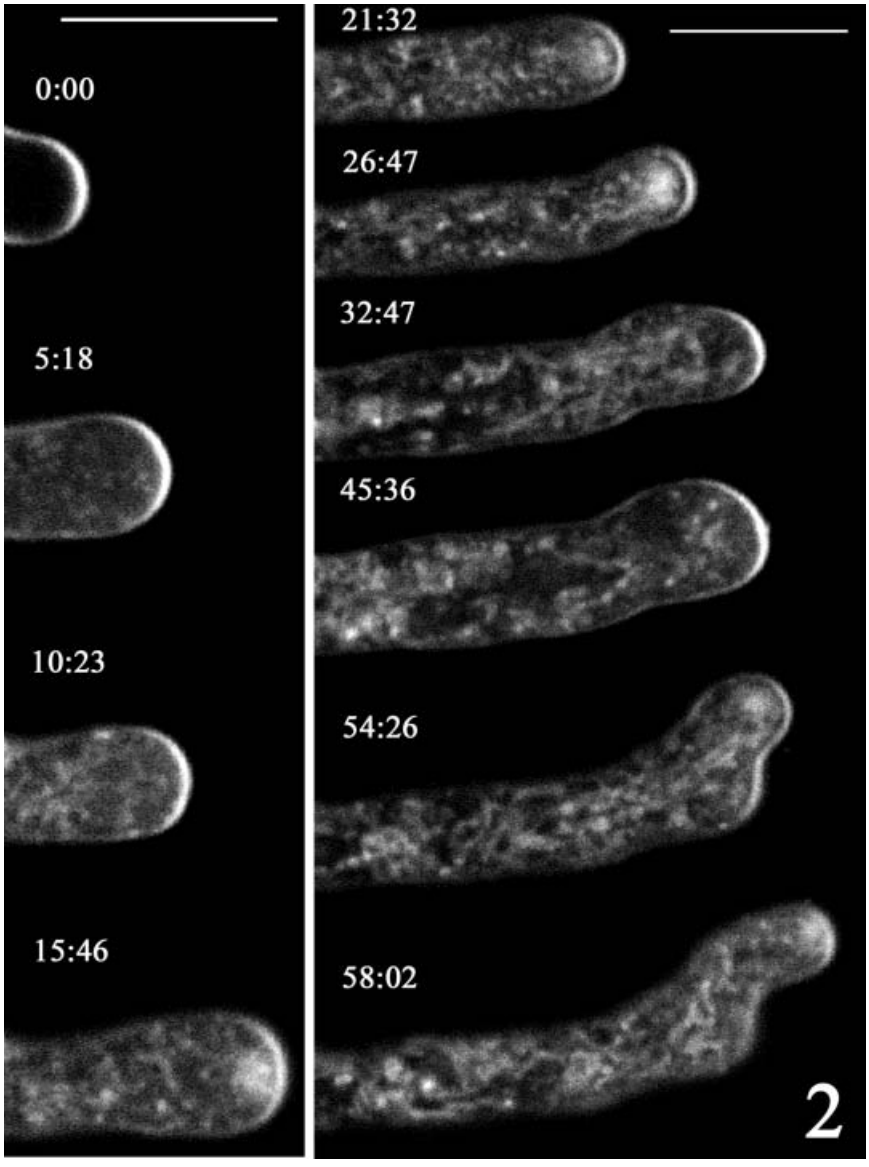

Fig. 2. *Puccinia graminis*. Confocal microscopy showing germ tube growth and behavior over a period of about 1 h. FM4-64 was added about 30 s before the first image was collected. The left panel shows staining of the Spk inside the germling. Note that the plasma membrane stains immediately (0 min). Small, stained cytoplasmic inclusions are readily visible after 10 min, and the Spk is clearly visible after 16 min. In the right panel disappearance and reappearance of the Spk are shown. Reappearance of the Spk is associated with resumed growth. In each image the germ tube was optically sectioned in a region adjacent to the substratum where the Spk, when present, is located. Bars: 10 μm

Behavior of the Spitzenkörper

The size, shape, and fluorescence intensity of the stained vesicle cloud associated with the Spk was very dynamic and could change over short time periods. The latter is shown in images captured at intervals of 5 s (Fig. 3b) showing small changes in position and shape of the Spk.

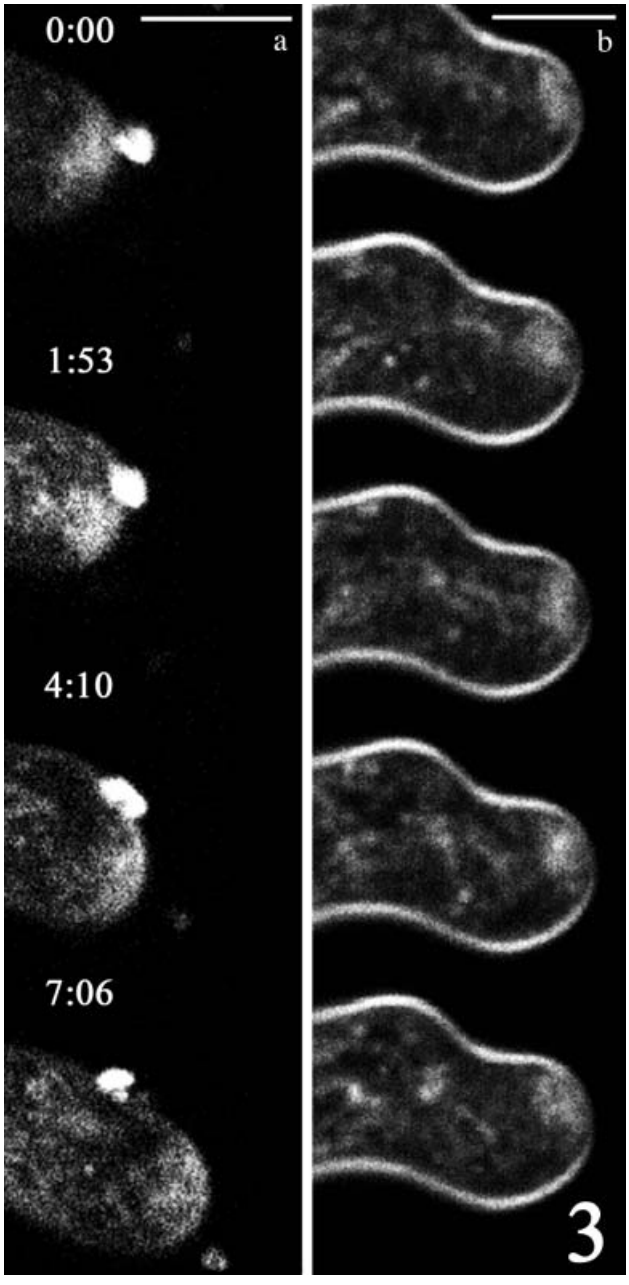

Fig. 3a, b. Confocal microscopy of growing germ tubes stained with FM4-64. **a** *Uromyces vignae*. Behavior of a germ tube tip when it encounters a small fluorescent particle and grows around it. Note the change in position and morphology of the Spk and cell expansion at its new position. **b** *Puccinia graminis*. Variations in shape and position of the Spk are illustrated during a 25 s period with time intervals of 5 s. Bars: 5 μm

Bleaching of FM4-64 inside the fungal cell only occurs after irradiation times much longer than those used in this study and is negligible during the acquisition of 5 consecutive scans with a pause of 5 s between them. In fact, the fourth image of the series depicted in Fig. 3b shows the most intensive fluorescence.

On flat surfaces, germ tubes exhibited frequent changes in growth direction. Germ tubes of *P. graminis* were more prone to branch formation than those of *U. vignae*. The movement of the Spk to a new location in the apical dome of the germ tube preceded outgrowth of the germ tube in that region. This intimate relationship between the behavior of the Spk and subsequent changes in growth direction was also observed when external particles were encountered by the germling (Fig. 3a). Upon contact of the germ tube with the particle, the Spk shifted to one side. This was followed by bulging and new growth of the germ tube on that side. Another feature often observed was that the germ tube would transiently cease polarized growth and then became more swollen before exhibiting polarized growth again (Fig. 2, right panel). Cessation of polarized growth was always correlated with the disappearance of the Spk, and resumption of growth was always correlated with its reappearance. Over the course of the observation period, the growth rate of the germ tubes began at 0.9 μm/min, dropped to zero, and then rose to 0.9 μm/min again.

Spitzenkörper behavior during appressorium and penetration peg differentiation

The initial stages of appressorium differentiation of *U. vignae* over an about 0.5 μm high, microfabricated step are shown in Fig. 4. During contact of the hyphal tip with the step, the shape of the Spk changed; it was easily recognizable before and after the encounter (Fig. 4a, b), but could not be recognized at later stages of development (4.5 min following contact, Fig. 4c). At this time, the germ tube tip swelled (Fig. 4c–e). After 39 min an appressorium was nearly fully expanded (Fig. 4f). Near to the glass surface it had undergone clear bipolar growth along the artificial step (Fig. 4f, right image), which clearly is a contact-mediated response (Kwon and Hoch 1991, Collins et al. 2001). In the left image of Fig. 4f two nuclei are visible as nonstaining regions. Nuclei did not take up dye, even after long periods of staining. During later stages of development, after completion of mitosis, four nuclei were observed inside the appressorium. Subsequently, a penetration peg started to develop on the underside of the mature appressorium. In Fig. 5 this stage is depicted and the image shows a diffusely stained region which stained most intensively

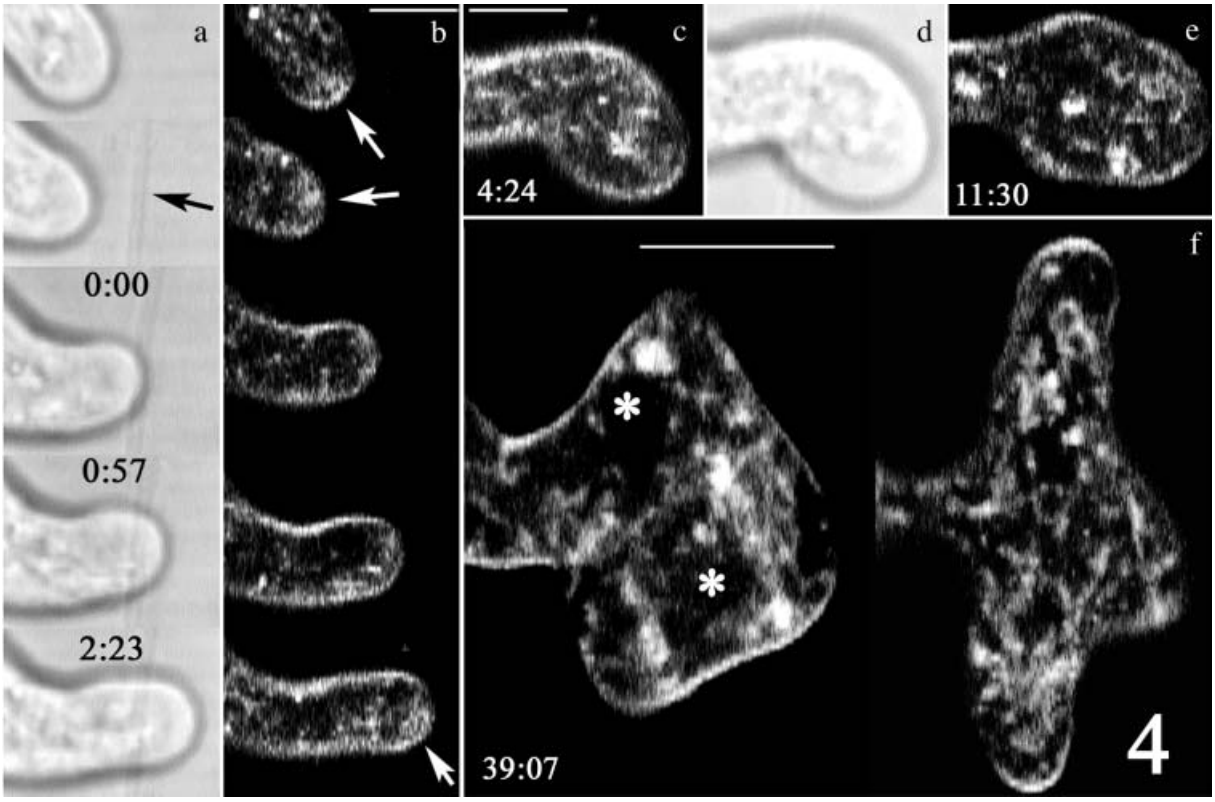

Fig. 4a–f. Confocal microscopy of differentiating appressoria of *U. vignae* stained with FM4-64. In order to prevent the induction of germ tube swelling as a result of confocal microscopy, this germ tube was imaged by the fast-scanning, low-resolution F4 setting of the confocal microscope. **a** and **b** Growth of a germ tube over a differentiation-inducing step (indicated with a black arrow). The Spk can be recognized clearly during three stages (white arrows). Times (in min) indicate the period after the germ tube encountered the step. **c–e** Two stages in the swelling of a germ tube after cessation of growth. **d** Transmission image corresponding to the fluorescence image **c**. **f** Nearly fully formed appressorium shown above (left) and very near (right) to the glass surface. Two nuclei are visible as nonstaining areas (asterisks) in the left image. Note the clear bipolar growth of the appressorium along the step. Bar: b and c, 5 μm; f, 10 μm

near the site of contact with the glass substratum (Fig. 5, right image).

Discussion

In this study we have used the term Spitzenkörper to describe the stained apical vesicle cloud of rust germ tubes. In a number of aspects the "apical vesicle cluster" behaves like the typical Spk described for many different fungal species (Girbardt 1957, López-Franco and Bracker 1996). These resemblances include its presence within growing apices, its behavior prior to changes in growth direction (see also Hoffman and Mendgen 1998), and the predominance of vesicles as structural components (Kwon and Hoch 1991).

Other features of rust Spk distinguish them from Spk of vegetative hyphae of most other fungi. Firstly, rust germ tube Spk lack a differentiated "core" region. Although Spk organization differs among fungal genera (López-Franco and Bracker 1996), a core region is seen in different ascomycetes and basidiomycetes (Grove and Bracker 1970, Howard and Aist 1979, Roberson and Fuller 1988) by both light and electron microscopy. In previous studies on germ tubes of the rust *U. appendiculatus*, a core region was not observed (Hoch and Staples 1983, Kwon et al. 1991).

A second feature distinguishing Spk in rusts is that they are located asymmetrically within the hyphal tip of the germ tube. This was observed earlier in germ tubes of *U. appendiculatus* that were fixed by rapid freezing and observed by electron microscopy (Kwon and Hoch 1991) and has been confirmed in this study in living and growing rust germ tubes of *U. vignae* and *P. graminis*. Remarkably, this feature was also observed by electron microscopy in germ tubes of two other plant pathogenic fungi, namely, *Cochliobolus sativa* (Clay et al. 1996) and *Magnaporthe grisea* (Bourrett and Howard 1989).

A third feature of rust Spk is their highly dynamic behavior during short time spans and after an encounter of extracellular features and that it appears and reappears virtually spontaneously during germ tube development. Furthermore, the Spk disappears during early stages of

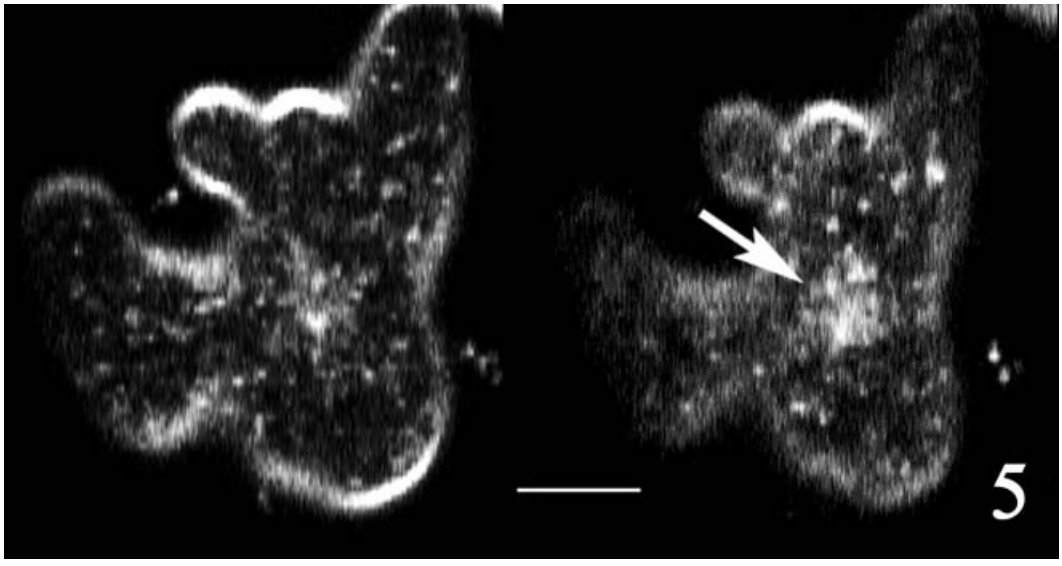

Fig. 5. Two optical sections of a fully formed appressorium of *U. vignae* showing a clear accumulation of vesicles (arrow) near the position where the penetration peg will be formed. The right image is closest to the glass surface. Bar: 5 μm

appressorium formation, but a similar-looking structure reappears when the penetration peg is formed.

The Spk of rust fungi stains markedly more slowly after addition of the dye FM4-64 than do those of other fungal species such as *Neurospora crassa* and *Rhizoctonia solani* (Fischer-Parton et al. 2000). Hyphae of *R. solani* grew approximately twice as fast as rust germ tubes but exhibited a much faster staining Spk than rusts. The structure was visible after 2 min in case of *R. solani* and after 10 min with the rust fungi (J. Dijksterhuis and N. D. Read unpubl. results). This may indicate that the endomembrane system in rust germlings differs from that of vegetative hyphae. The asymmetric location of the Spk and its behavior suggest a special sensitivity of the Spk for external topographical features and may play an important role in the process of touch sensing (Hoch et al. 1987, Collins and Read 1997). This role may include the redirection of germ tube growth as observed on leaves and artificial substrates. The reorientation may arise from the deposition of cell wall material, which follows the shape of the topography. As an example, confocal microscopy showed that *P. graminis* cell walls were deposited between ridges (on polystyrene wafer replicas) that were only a few micrometers apart (data not shown).

Touch sensing also leads to appressorium formation over stomata, which is regarded as a more extensive differentiation process. The swelling of the hyphal tip is followed by continuous enlargement, nuclear division, contraction of the cytoplasm into the appressorium compartment, and septum formation (Kwon et al. 1991). We observed a time course for differentiation in *U. vignae* similar to that described for *U. appendiculatus* (Kwon and Hoch 1991). A distinct Spk was not evident once the germ tube started to swell. An accumulation of vesicles, however, may reappear during later stages of polarized growth (as the bipolar outgrowth in Fig. 4 or the formation of an elongated appressorium of *P. graminis* after a temperature shock reported by Staples and Hoch [1983]). This is illustrated by the clear presence of an apparent vesicle cloud prior to the formation of a penetration peg.

The regular appearance and disappearance of the Spk suggest that the responsiveness of the germ tube to topographies is related to a relatively "instable" organization of the Spk.

Acknowledgments

My work was supported by a grant from the Leverhulme Trust to Nick Read. I thank Nick Read for valuable discussions and help during the preparation of the manuscript and Richard Summerbell for critical reading of the manuscript. Thanks are also due to Alan Gundlach and Tom Stevenson at the Department of Electrical Engineering of the University of Edinburgh for the production of microfabricated steps on glass coverslips, Sabine Fischer and Patrick C. Hickey for helpful discussions and technical advice, and to Rosamaria López-Franco and Charles Bracker for helpful discussions.

References

Betz WJ, Mao F, Smith CB (1996) Imaging exocytosis and endocytosis. Curr Opin Neurobiol 6: 365–371

Bourett TM, Howard RJ (1989) In vitro development of penetration structures in the rice blast fungus *Magnaporthe grisea*. Can J Bot 68: 329–342

Clay RP, Enkerli J, Fuller MS (1994) Induction and formation of *Cochliobolus sativus* appressoria. Protoplasma 178: 34–47

Collins TJ, Read ND (1997) Appressorium induction by topographical signals in six cereal rusts. Physiol Mol Plant Pathol 51: 169–179

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

Girbardt M (1957) Der Spitzenkörper von *Polystictus versicolor* (L.). Planta 50: 47–59

– (1969) Die Ultrastruktur der Apikalregion von Pilzhyphen. Protoplasma 67: 413–441

Grove SN, Bracker CE (1970) Protoplasmic organisation of hyphal tips among fungi: vesicles and Spitzenkörper. J Bacteriol 104: 989–1009

Heath IB (ed) (1990) Tip growth in plant and fungal cells. Academic Press, San Diego

Hoch HC, Staples RC (1983) Ultrastructural organisation of the non-differentiated urediospore germling of *Uromyces phaseoli* variety *typica*. Mycologia 75: 795–824

– – Whitehead B, Comeau J, Wolf ED (1987) Signaling for growth orientation and cell differentiation by surface topography in *Uromyces*. Science 235: 1659–1662

Hoffmann J, Mendgen K (1998) Endocytosis and membrane turnover in the germ tube of *Uromyces fabae*. Fungal Genet Biol 24: 77–85

Howard RJ (1981) Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkörper, cytoskeleton and endomembranes after freeze-substitution. J Cell Sci 48: 89–103

– Aist JR (1979) Hyphal tip cell ultrastructure of the fungus *Fusarium*: improved preservation by freeze substitution. J Ultrastruct 66: 224–234

Kwon YH, Hoch HC (1991) Temporal and spatial dynamics of appressorium formation in *Uromyces appendiculatus*. Exp Mycol 15: 116–131

– – Aist JR (1991) Initiation of appressorium formation in *Uromyces appendiculatus*: organization of the apex, and the responses involving microtubules and apical vesicles. Can J Bot 69: 2560–2573

López-Franco RM, Bracker CE (1996) Diversity and dynamics of the Spitzenkörper in growing hyphal tips of higher fungi. Protoplasma 195: 90–111

– Bartnicki-Garcia S, Bracker CE (1994) Pulsed growth of fungal tips. Proc Natl Acad Sci USA 91: 12228–12232

Read ND, Hickey PJ (2001) The vesicle trafficking network and tip growth in fungal hyphae. In: Geitmann A, Cresti M, Heath IB (eds) Cell biology of plant and fungal tip growth. IOS Press, Amsterdam, pp 137–148

– Kellock LJ, Knight H, Trewavas AJ (1992) Contact sensing during infection by fungal pathogens. In: Callow JA, Green JR (eds) Perspectives in plant cell recognition. Cambridge University Press, Cambridge, pp 137–172

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

Staples RC, Hoch HC (1983) Ultrastructural organization of the non-differentiated uredospore germling of *Uromyces phaseoli* variety *typica*. Mycologia 75: 795–824

Vida TA, Emr SD (1995) A new vital stain for visualising vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 128: 779–792

Wedlich-Söldner R, Bölker M, Kahmann R, Steinberg G (2000) A putative endosomal t-SNARE links exo- and endocytosis in the phytopathogenic fungus *Ustilago maydis*. EMBO J 19: 1974–1986

Wynn WK (1976) Appressorium formation over stomates by the bean rust fungus: response to a surface contact stimulus. Phytopathology 66: 136–146