Protoplasma (2002) 219: 13–22

PROTOPLASMA

Cytoskeletal responses during early development of the downy mildew of grapevine (*Plasmopara viticola*)

M. Riemann[1,*], **C. Büche**[2], **H.-H. Kassemeyer**[2], and **P. Nick**[1]

[1] Institut für Biologie II, Albert-Ludwigs-Universität Freiburg, Freiburg
[2] Staatliches Weinbauinstitut, Freiburg

Received October 4, 2001
Accepted October 23, 2001

Summary. A host-free system was established to induce the early development of the obligate biotrophic pathogen *Plasmopara viticola*, the downy mildew of grapevine. This system was used to study cytoskeletal responses during encystation and germ tube formation. During these processes, both the actin and the tubulin cytoskeleton show a stage-specific pattern of distribution. Elimination of the cytoskeleton by the actin drug latrunculin B and the microtubule drug ethyl-N-phenyl-carbamate did not affect the release of mobile zoospores from the sporangia, nor the encystation process, but efficiently inhibited the formation of a germ tube. The data are discussed with respect to a role of both actin and microtubules for the establishment of the cell polarity guiding the emergence and the growth of the germ tube.

Keywords: *Plasmopara viticola*; Latrunculin B; Ethyl-N-phenyl-carbamate; Cytoskeletal response; Polarity; Germ tube.

Introduction

Plasmopara viticola is one of the most important pathogens of *Vitis vinifera* in moderate climates. Its infection cycle is characterized by fast transitions between different developmental stages (for detailed description, see Müller and Sleumer 1934). For successful infection, the zoospores of *P. viticola* have to reach the appropriate host cell, the guard cells on the lower surface of the leaf. They subsequently encyst and develop a germ tube that penetrates into the respiratory cavity. The germinating spore is thus behaving as a typical tip-growing cell.

In general, tip growth requires a vigorous transport of vesicles, a polarity guiding this transport, and the allocation of resources to the growing tip. The cytoskeleton can meet at least the first two of these requirements for tip growth. Microtubules and filamentous actin (F-actin) are both polar structures that can serve as tracks for the transport of vesicles carrying cell wall components or enzymes to the actively growing zone (Gow 1995, Heath 1995). Furthermore, there exist molecular motors, such as kinesins and dyneins for microtubules and myosins for F-actin, that can move along these tracks and carry specific cargoes (for a review, see Nick 1999). Numerous examples demonstrate that cell polarity can be disturbed by treatment with cytoskeletal drugs. For instance, the axis of root growth in *Arabidopsis thaliana* (Baskin et al. 1994) and the induction of polarity by light in zygotes of fucoid algae (Kropf et al. 1998) are affected upon the elimination of F-actin or microtubules. In the oomycete *Phytophthora cinnamomi*, zoosporogenesis and the correct distribution of vesicles and organelles have been attributed to microtubules (Hyde and Hardham 1993), and the participation of the cytoskeleton in sporangium development, zoosporogenesis, or hyphal tip morphogenesis has been described for other oomycetes as well (e.g., Harold and Harold 1992, Temperli et al. 1990, Heath et al. 2000).

The third prerequisite of tip growth is the continuous allocation of energy and genetic information into the growing region over a distance that is too large to be bridged by mere diffusion. Again, it is the cytoskeleton that drives this process: Microtubules seem to be involved in nuclear movement in *Phytophthora cinnamomi* (Hyde and Hardham 1993),

* Correspondence and reprints: Institut für Biologie II, Albert-Ludwigs-Universität Freiburg, Schänzlestrasse 1, 79104 Freiburg, Federal Republic of Germany.

whereas in *Allomyces macrogynus* this task is brought about by actin microfilaments (Lowry et al. 1998). Mutants where the redistribution of nuclei into the hyphae is impaired, such as the *nud* mutants of *Aspergillus nidulans* or the *ro* mutants of *Neurospora crassa*, demonstrate the importance of nuclear migration for tip growth (for a review, see Morris 2000). These mutants grow slowly, branch more frequently, and sporulate rarely. In most of these lines, the mutation affected genes that either code for motor proteins or other cytoskeleton-associated proteins.

To investigate the role of the cytoskeleton during early development of *P. viticola*, we studied the dynamics of microtubules, actin filaments, and vesicles. We developed a standardized system to induce the release of zoospores and the formation of a germ tube in the abscence of the host. To identify developmental key events that depend on the cytoskeleton, we administered specific cytoskeletal drugs inhibiting the polymerization of tubulin (such as ethyl-N-phenyl-carbamate [EPC]) and actin (such as latrunculin B). Our findings suggest a role for actin and tubulin for cell polarity in the encysted spores during the formation of a germ tube related to vesicle transport. We additionally demonstrated that spores with a different number of nuclei exist and that the nucleus moves during germination.

Material and methods

Plant material

As host plant for the cultivation of *Plasmopara viticola* served cuttings of the grapevine cultivar Müller-Thurgau (*Vitis vinifera* L. cv. Müller-Thurgau). This cultivar is very susceptible for downy mildew of grapevine. The plants were grown in the greenhouse in pots. To ensure a minimal day-length of 16 h, the plants were additionally irradiated with lamps (400 W, Vialox NAV-T (SON-T); Osram, Munich, Federal Republic of Germany).

Pathogen material

Plasmopara viticola (Berk. & Curtis) Berl. & DeToni was cultivated in the greenhouse on the host plant. For the infection of the plants, the lower surface of the leaves was sprayed with an aequous suspension of sporangia by means of a chromatography sprayer (Ecospray; Roth, Karlsruhe, Federal Republic of Germany) until the suspension was dripping off. A plastic bag was rinsed with water, and the entire plant covered with the bag overnight.

After 4 or 7 days of incubation in the greenhouse, sporulation could be triggered by covering the preinfected plant with a humid plastic bag overnight. Successful infection became manifest as a white, powdery coating on the lower leaf surface. This coating, consisting of sporangiophores and sporangia was air dried and transferred into a plastic tube. It can be stored at –20 °C; however, for the physiological experiments only freshly harvested material was utilized.

Time course studies on the early development of P. viticola

Sporangia of *P. viticola* were suspended to homogeneity in 10 ml of destilled water and transferred into a petri dish. After 3 h of incubation in white light (5 W/m^2) in a phytotron (Type RTL 4; Ehret GmbH, Emmendingen, Federal Republic of Germany) at 22 °C, encystment and germination of the zoospores was induced by addition of 10 mM sodium chloride. The suspension was returned to the phytotron. Encystation occurred virtually instantaneously upon addition of sodium chloride, and 1 h later numerous spores had generated a germ tube.

To quantify the time course of the first developmental stages of *P. viticola* in the absence of the host plant, the frequency of different stages was scored under a light microscope in aliquots of 50 µl with a haematocytometer for a defined volume (Fuchs-Rosenthal; Thoma, Freiburg, Federal Republic of Germany).

To standardize the quantity of the release of zoospores from the sporangia, the maximal number of zoospores was defined as 100% and the data obtained at the other time points were calculated relative to this standard. To standardize the scores of the subsequent stages (zoospores, encysted spores, and spores forming a germ tube), the relative frequency of each stage was calculated in relation to the total number of spores. The curves represent the data from at least 4 individual time course experiments comprising more than 17000 individual spores.

Inhibitor treatments

Ethyl-N-phenyl-carbamate (EPC) (Wako Pure Chemicals Ltd., Tokyo, Japan; 100 mM stock solution in ethanol) and latrunculin B (Calbiochem, Nottingham, U.K.; 10 mM stock solution in dimethyl sulfoxide [DMSO]) were added in different concentrations at the beginning of the experiment, when the sporangia were suspended in water. The formation of germ tubes was induced by sodium chloride and quantified as described above.

Visualization of microtubules and actin microfilaments

Microtubules and microfilaments were stained in a staining chamber consisting of a dialysis tube with fitting clips. For the staining of microtubules a 5 cm long membrane with 300 kDa exclusion size (Spectra/Por; Roth) and for the staining of microfilaments a 5 cm long membrane with 14 kDa exclusion size (Visking, Typ 27/32; Roth) was used. The dialysis tube was closed with a clip at one end, and a micropipette tip (volume, 1 ml; Roth) was inserted into the open end at the opposite side such that it could be used as a funnel to fill the chamber. The chamber was fixed within a glass beaker (volume, 50 ml) such that the micropipette tip was located at the upper side. Now, the chamber could be filled with antibody or dye solutions, and the specimen could be washed by filling the beaker with washing buffer that penetrated through the dialysis membrane.

For the staining of microtubules, cells were fixed in 3.7% paraformaldehyde, 5% DMSO, and 2 µl of glutaraldehyde (50%, v/v, in H_2O) in a total volume of 200 µl for 1 h under rotation at room temperature. Different concentrations of fixative (glutaraldehyde), DMSO, and detergents (Nonidet P-40) were compared to find the optimal concentration (data not shown). The fixed material was transferred into the staining chamber, incubated in microtubule-stabilizing buffer (MSB) (50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 5 mM EGTA, 1 mM $MgSO_4$, 1% [w/v] glycerol, 0.25% Triton X-100, pH 6.9) and washed for 30 min. The MSB was replaced twice during this period. Then, unspecific binding sites were blocked by incubation with 5% horse normal serum in Tris-buffered saline (TBS) (150 mM NaCl, 20 mM Tris, 0.25% Triton X-100, pH 7.4) for 20 min. The blocking solution was washed out for 5 min with TBS. The anti-α-tubulin antibody (Amersham, Little Chalfont, U.K.) was

added to the staining chamber in a dilution of 1 : 50. The suspension was incubated for 1 h at 37 °C, subsequently washed 3 times for 5 min in TBS and incubated with the secondary antibody (anti-mouse, developed in goat, coupled to fluorescein isothiocyanate; Sigma, Neu-Ulm, Federal Republic of Germany) in a dilution of 1 : 25. The whole chamber was incubated for 1 h at 37 °C and subsequently washed 5 times for 5 min in TBS.

For the staining of actin, cells were fixed for 15 min at room temperature in MFB diluted from a 5× stock solution (500 mM K_2HPO_4, 500 mM KH_2PO_4, pH 7.3, 500 mM KCl, 1.25% Triton X-100) and 1.85% paraformaldehyde (diluted from a stock solution of 18.5% in MFB). For the determination of optimal staining conditions different concentrations of fixative and detergents were compared (data not shown). The fixed cells were transferred to the staining chamber and washed for 15 min in MFB. To stain the cells, 330 nM Texas Red-Phalloidin in MFB (Molecular Probes, Eugene, Oreg., U.S.A.) were filled into the dialysis tube and the cells incubated for 30 min at room temperature in the dark. Subsequently, cells were washed for 30 min in MFB.

Visualization of vesicles and nuclei

To stain vesicles, a suspension of spores was mixed with 10 µg of rhodamine-G6-chloride (Molecular Probes) per ml and diluted with water from a stock at a concentration of 1 mg/ml in DMSO that was stored at –20 °C. The cells were transferred to a slide that had been coated with Meyer's adhesive (1 volume egg white, 1 volume glycerol, 1% [w/v] sodium salicylate), and incubated for 10 min at room temperature. The staining solution was removed with a paper towel and replaced by water. This washing step was repeated.

Nuclei were stained with 10 µg of propidium iodide (Sigma) diluted with water from a stock at a concentration of 1 mg/ml in DMSO that was stored at – 20°C, an intercalating dye of DNA (Arndt-Jovin and Jovin 1989). The samples were treated as described for vesicle staining.

Confocal microscopy

The stained cells were mounted on slides that had been coated with Meyer's adhesive and were examined with a confocal microscope (Leitz DM RBE coupled to a True Confocal Scanner; Leica TCS 4D, Bensheim, Federal Republic of Germany) using an argon-krypton laser with excitation at 488 nm, a beam splitter at 510 nm, and an emission filter at 515 nm wavelength for the visualization of microtubules, whereas microfilaments and the vesicles were visualized with an excitation at 568 nm, a beam splitter at 578 nm, and an emission filter at 590 nm. To obtain a high signal-to-noise ratio, a line averaging algorithm based on 16 or 32 individual scans per image was applied. The preparations were examined with an oil immersion objective (magnification, 100) and digitized with the Scanware software (Leica, Heidelberg, Federal Republic of Germany). Images were processed with Photoshop 6.0 (Adobe Systems Europe, Edinburgh, U.K.) and Designer 7.0 (Micrografix Deutschland GmbH, Unterschleissheim, Federal Republic of Germany).

Results

Host-free system to induce the release of zoospores and germ tube formation in P. viticola

To observe the early stages in the vegetative life-cycle of *P. viticola*, a host-free system had to be established at first. Figure 1 A shows schematically the sequence of events from the release of zoospores over encystment to germ tube formation. The release of zoospores could be induced by resuspending the sporangia in water at 22 °C. The average release time was 2.5 h after induction, complete release of the population was reached within 4 h (Fig. 1 B). After the zoospores have been released, further development could be triggered by addition of 10 mM NaCl (Fig. 1 A), whereas without the addition of salt only a small percentage of zoospores continued to develop (data not shown). Upon addition of salt, the frequency of mobile zoospores decreased to 50% within 10 min (Fig. 1 C), whereas the frequency of encysted spores simultaneously increased to about 35% (Fig. 1 D). These encysted spores formed a germ tube. About 40 min after induction with NaCl, half of the encysted spores had produced a germ tube, the maximum was reached about 80 min after induction with salt (Fig. 1 E). An intriguing observation was that the entire cytoplasm was translocated into the germ tube, leaving the residual spore empty (Fig. 1 F). For longer time intervals, the frequency of spores with germ tubes decreased again. This correlates with the observation that no subsequent developmental steps could be observed in the absence of a host leaf.

Distribution of cytoskeletal structures and vesicles during germ tube formation

The patterns of microtubules, actin, and vesicles were visualized in encysted spores (Fig. 2 A–C) and in spores that had formed a germ tube (Fig. 2 D–F). A range of different protocols for fixation and permeabilization were compared (data not shown) for the visualization of reliable, stage-specific cytoskeletal patterns.

In encysted, spores, fine actin microfilaments connect small granules of actin in a continuous network throughout the cell in encysted spores (Fig. 2 A). At one pole of the cell, possibly at the site where the germ tube will emerge, actin accumulates in a large patch (Fig. 2 A). Similarly, the major proportion of the tubulin signal is organized in form of larger, round patches that accumulate in one hemisphere of the cell and are connected by scarce microtubules (Fig. 2 B). Numerous vesicles are visible in encysted spores (Fig. 2 C), but they are distributed more or less homogeneously throughout the cell.

These patterns change during formation of a germ tube (Fig. 2 D–F). The actin granules gradually move from the cell body into the emerging germ tube and

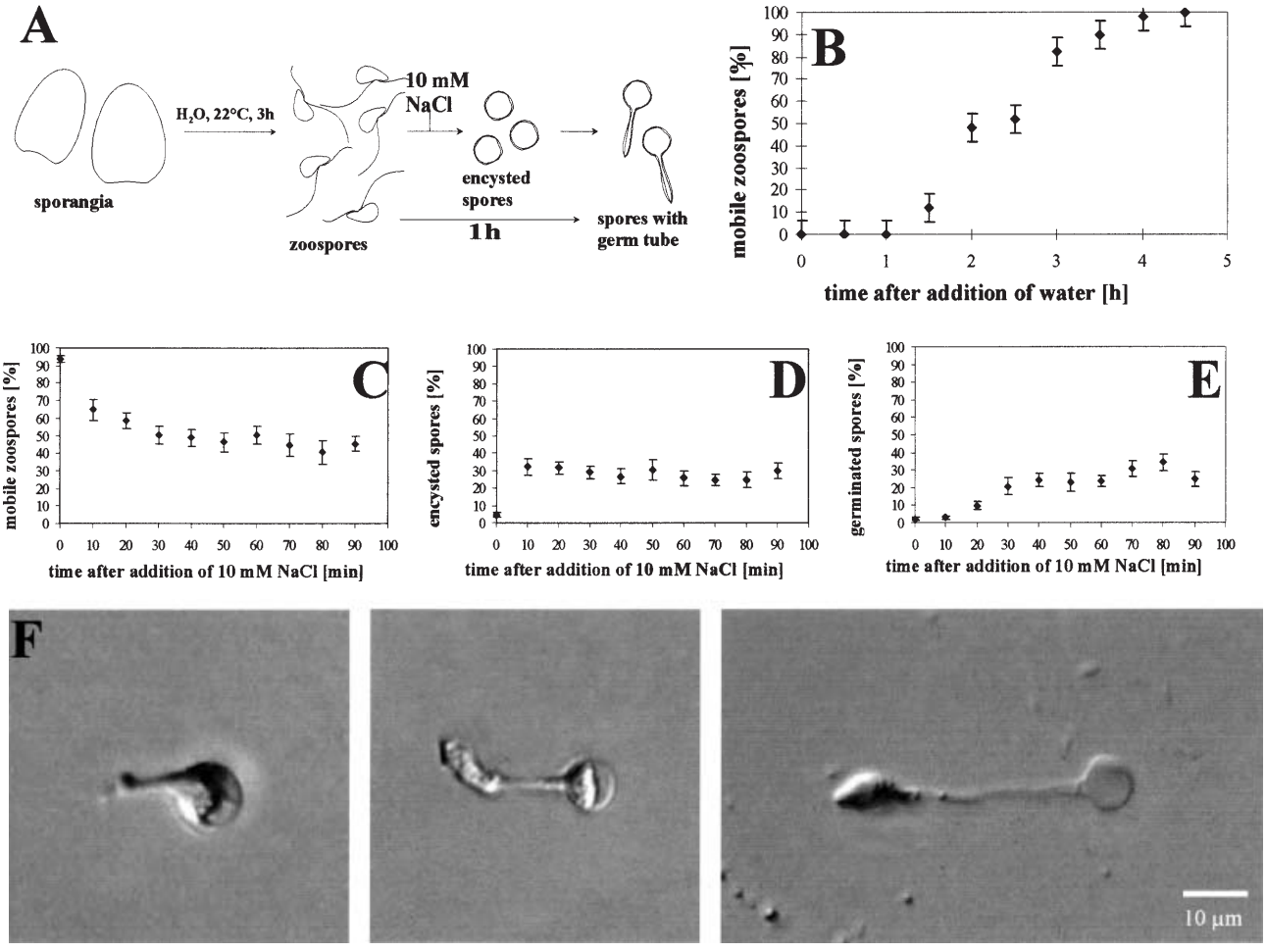

Fig. 1 A–F. Host-free system to study the early development of *P. viticola*. **A** Schematic representation of the experimental system. Zoospores are released within 3 h of incubation in water and can be induced to encyst and germinate by addition of 10 mM NaCl. **B–E** Time courses for the developmental stages in this host-free system. The maximum value of zoospores was defined as 100% in **B** (n = 3779), in **C–E** the frequency of the developmental stages was calculated as frequency of the total population of spores (n = 17433). **C** Disappearance of mobile zoospores after addition of 10 mM NaCl. **D** Encystation of spores. **E** Germ tube formation. **F** Light-microscopical view of germinating spores showing progressive stages of germ tube growth. The cytoplasm is displaced into the germ tube

are interconnected by long microfilaments (Fig. 2E). At the tip of the germ tube a cap of actin is visible (Fig. 2E, inset). The tubulin signal is conspicuously depleted from the hemisphere opposite to the germ tube, whereas the hemisphere where the germ tube emerges, especially the region around the nucleus, is strongly labeled (Fig. 2F). The cell body contains filamentous structures which reach into the germ tube (Fig. 2F). In the germ tube the tubulin signal is concentrated in bulges, where the direction of growth has changed. The vesicles predominant in encysted spores (Fig. 2C) are replaced by a diffuse network (Fig. 2D) in the cell body and strong accumulations in the bulges or the tip of the germ tube or diverging side branches of the tube.

Influence of cytoskeletal drugs on spore release, encystation, and germ tube formation

To study the participation of cytoskeletal elements in the process of encystation and germ tube formation of *P. viticola*, we examined the effect of EPC, an inhibitor of microtubule assembly, and of latrunculin B, a potent inhibitor of actin assembly. Zoospores were released in the presence of different concentrations of these drugs. After 3 h, sodium chloride was added to a concentration of 10 mM to induce germ tube formation, and the number of spores in the different developmental stages was followed over time.

EPC, an inhibitor of tubulin polymerization (Fujimura et al. 1992), was found to inhibit the

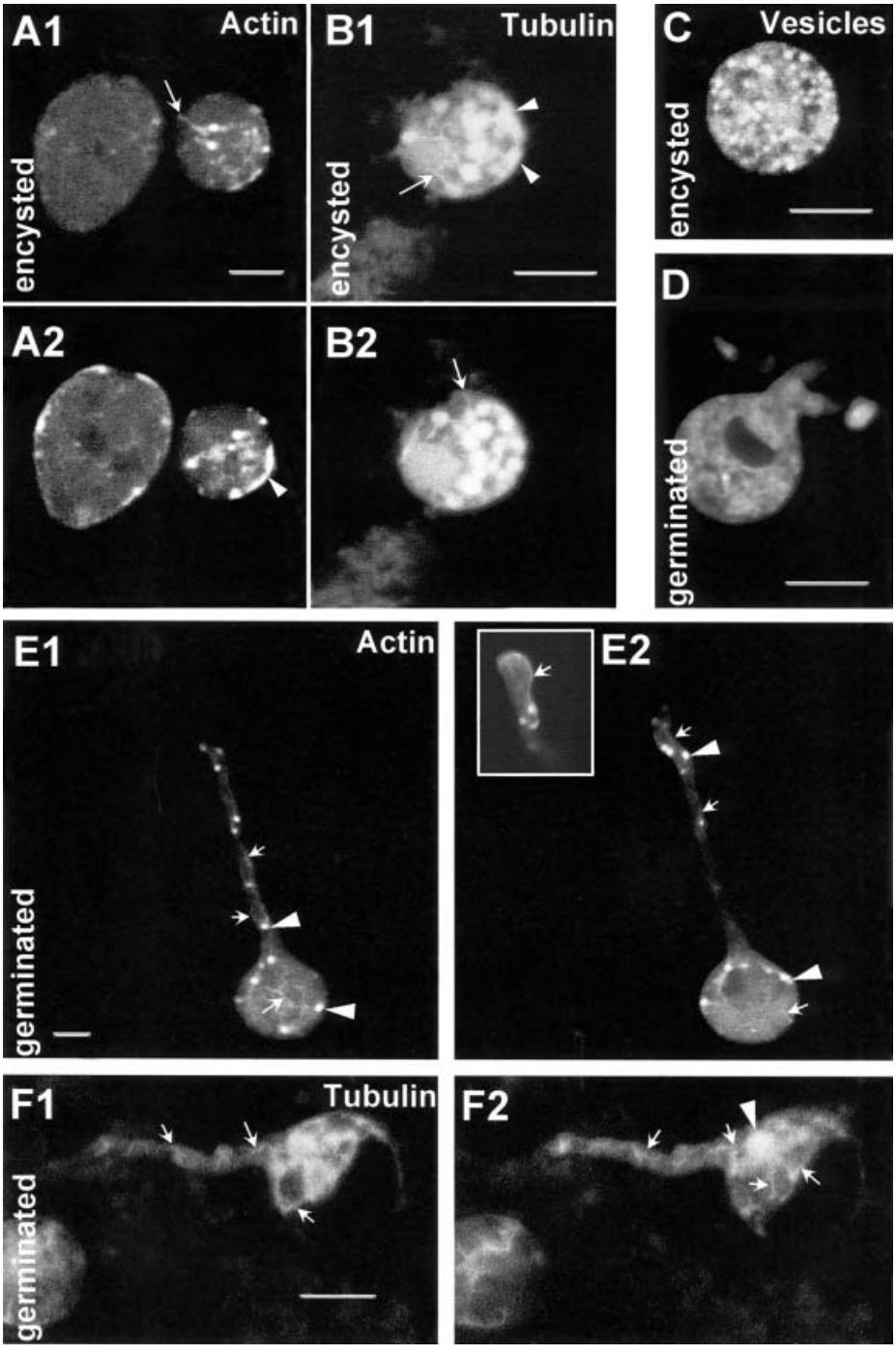

Fig. 2. Cytoskeletal and vesicle structures observed during encystation (**A–C**) and germ tube formation (**D–F**) in *P. viticola* by confocal laser scanning microscopy. Bar: 5 µm. **A** Visualization of actin in an encysted spore. Two confocal sections (A1 and A2) shown for one spore. The arrowhead indicates accumulation of actin patches at one pole of the cell, the arrow indicates a microfilament bundle. **B** Visualization of tubulin in an encysted spore. Two confocal sections (B1 and B2) shown for one spore. The arrowhead indicates accumulation of tubulin patches in one hemisphere of the spore, the arrows indicate microtubules. **C** Visualization of vesicles in an encysted spore. Numerous vesicles are distributed throughout the cell. **D** Visualization of vesicles in a germinated spore. In the residual cell, the vesicles are distributed in a network, whereas they accumulate in the tip of the germ tube. **E** Visualization of actin in a germinated spore. Two confocal sections (E1 and E2) shown for one spore. **Inset** Tip of the germ tube taken at a different *z*-level. Throughout the whole cell, actin is organized in plaquelike structures (arrowheads), which are connected by filaments (arrows). It accumulates in the tip of the germ tube (**inset**, arrow). **F** Visualization of tubulin in a germinated spore. Two confocal sections (F1 and F2) shown for one spore. Tubulin is concentrated in bulges of the germ tube and is forming filamentous structures in the tube (arrows). In the residual spore an accumulation of tubulin is visible near the base of the germ tube (arrowhead)

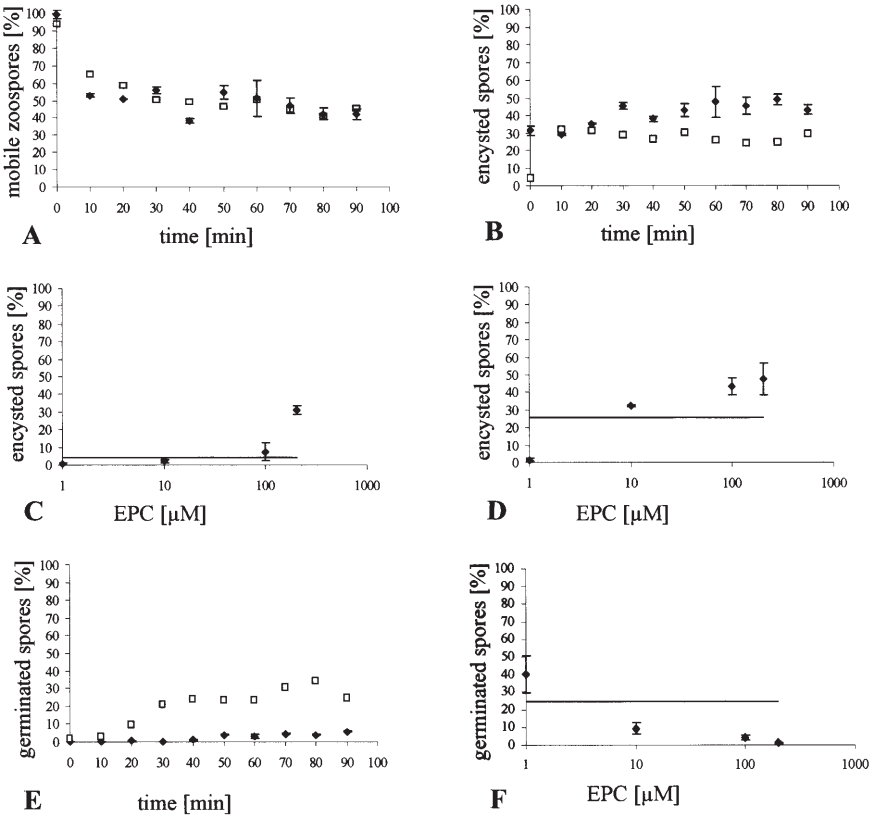

Fig. 3 A–F. Influence of the microtubule-eliminating drug EPC on early development of *P. viticola*. **A** Time course of zoospore release, **B** time course of encystation, **C** dose–response curve for spontaneous encystation prior to salt induction, **D** dose–response curve for encystation induced by 10 mN NaCl at 70 min after induction, **E** time course of germ tube formation, **F** dose–response curve for germ tube formation at 70 min after induction by 10 mM NaCl. □ Water-treated control, ◆ treatments with 200 µM of EPC. The solid lines in the dose–response curves represent the values observed in the water control. Each curve represents data from between 2000 and 3000 individual spores

formation of germ tubes at concentrations exceeding 1 µM (Fig. 3). However, there was no significant effect of the drug on the number of free zoospores even at 200 µM EPC, the highest concentration tested (Fig. 3 A). In contrast, the frequency of encysted spores increased significantly (Fig. 3 B, D). This increase was observed even without the addition of salt (Fig. 3 C). In contrast to encystation, the formation of germ tubes was inhibited very efficiently by EPC (Fig. 3 E): At a concentration of 200 µM EPC the portion of spores with germ tube reached only 5% (Fig. 3 F).

Latruculin B, an inhibitor of actin polymerization (Spector et al. 1983, 1989), accelerated the transition from free zoospores (Fig. 4 A) to encysted zoospores (Fig. 4 B, D) in response to salt. Encystation was elevated up to 90% measured 70 min after addition of sodium chloride (Fig. 4 B, D). Encystation was slightly elevated even in the absence of salt (Fig. 4 C) but not as pronounced as in the EPC-treated cells. Again, germ tube formation was inhibited very effectively (Fig. 4 E), in this case already by concentrations as low as 0.1 µM (Fig. 4 F).

Appearance of nuclei during early development of P. viticola

Nuclei were stained with propidium iodide and were observed to change in number, position, and shape. Encysted spores were found to exist in two types, either with one nucleus that was large and elliptical in shape (Fig. 5 B) or with two nuclei that were small and spherical (Fig. 5 A). In contrast, spores that had formed a germ tube contained always one nucleus (Fig. 5 C) that was ameboid in shape and migrating into the germ tube (Fig. 5 C).

Discussion

Induction of early development of infection stage of P. viticola in the absence of the host plant

When sporangia of *P. viticola* are incubated in water under suitable conditions, they release zoospores, but the subsequent stages of development are not initiated in the absence of the host plant. Only very few zoospores encyst and even less generate a germ tube. In suspensions that had been incubated up to 20 h, mainly envelopes of sporangia and zoospores were found (Cohen et al. 1999).

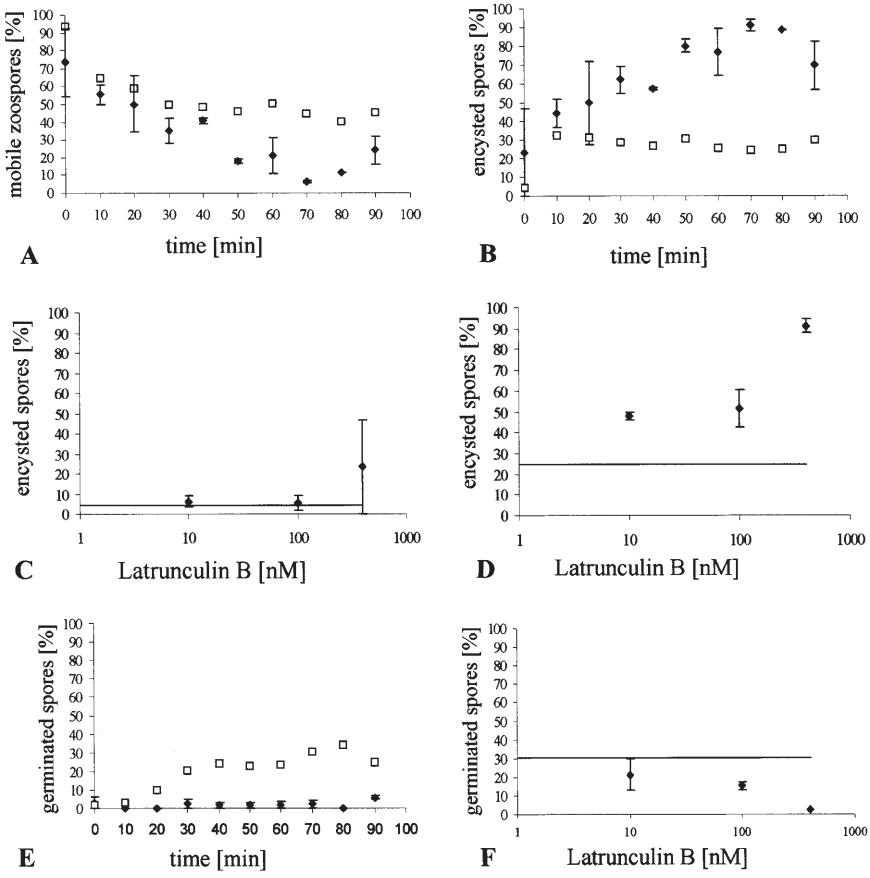

Fig. 4 A–F. Influence of the actin-eliminating drug latrunculin B on early development of *P. viticola*. **A** Time course of zoospore release, **B** time course of encystation, **C** dose–response curve for spontaneous encystation prior to salt induction, **D** dose–response curve for encystation induced by 10 mM NaCl at 70 min after induction, **E** time course for germ tube formation, **F** dose–response curve for germ tube formation at 70 min after induction by 10 mM NaCl. □ Water-treated control, ◆ treatments with 400 nM of latrunculin B. The solid lines in the dose–response curves represent the values observed in the water control. Each curve represents data from between 800 and 4500 individual spores

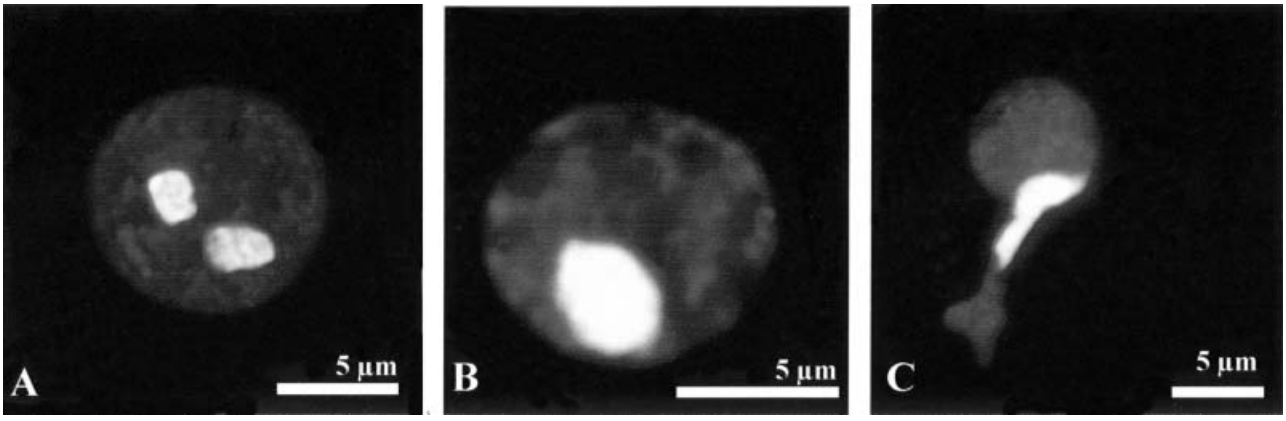

Fig. 5 A–C. Behavior of nuclei during encystation and germ tube formation. Nuclei were visualized with propidium iodide. Encysted spores were observed to possess either two small nuclei (**A**), or one large nucleus (**B**). Spores that had formed a germ tube were always mononucleate (**C**) with a large nucleus of ameboid appearance

We found a possibility to trigger some of the subsequent developmental steps by addition of 10 mM NaCl to the suspension. This might be taken as evidence that the real trigger is a change of osmotic potential. However, addition of mannitol cannot trigger encystation and germ tube development (S. Weis, Staatliches Weinbauinstitut, Freiburg, Federal Republic of Germany, unpubl. data), whereas sucrose is also an agent that can trigger germ tube formation (Cohen et al. 1999).

The possibility to study the development of zoospores in the absence of the host plant provides an essential prerequisite for the cytological investigation of the development during early infection stages. *Plas-*

mopara viticola cannot be cultivated on artificial medium, since hyphae develop only after contact with the host plant. Nevertheless, our host-free system has rendered the early steps of preinfection development accessible for cytological and physiological studies. The time course of early development (Fig. 1) revealed that the release of zoospores is a relatively slow process as compared to the subsequent developmental events such as encystation and formation of a germ tube.

The efficiency of this host-free system is not very high, however, because not all spores continue to develop. Although this system obviously can provide only a partial simulation of the natural situation, it is a useful tool to investigate the first developmental stages occurring during an infection cycle. Both, the proportion of encysted spores and the proportion of spores forming a germ tube do not exceed 30%. It is possible that there exist different subpopulations with deviating kinetic behavior, since natural populations and nonstandardized strains were used in the present study. However, the variation between different experimental series is relatively small, arguing against this possibility. This indicates that unknown factors presumably originating from the host plant enhance encystation and germ tube development.

Nuclei as flexible organelles during zoospore development

The behavior of nuclei during the development of spores of *P. viticola* is very dynamic with respect to position, shape, and number of nuclei. The most surprising situation was found in encysted spores (Fig. 5A, B). During this stage, spores with two nuclei coexist with spores that harbor only one nucleus. This observation can be explained by different scenarios. In the first scenario, the nuclei might fuse into one large nucleus, and this event might be the initiation of germ tube formation. However, oomycetes are strict diplonts (except for the gametangia and the gamete nuclei) and a fusion of nuclei at this stage of development cannot take place. This favors a second scenario where these two types might represent two parallel, independent developmental stages. In this case, it would be interesting to know if both types of spores are able to infect a leaf. The fact that we observed only one nucleus in germinated spores indicates that the binucleate spores cease to develop further. The difference in the number of nuclei is accompanied by a difference in shape: They are spherical in binucleate spores (Fig. 5A), elliptical in mononucleate spores (Fig. 5B), and ameboid in shape during the migration of the nucleus into the germ tube (Fig. 5C). In general, nuclear migration is an essential event in fungal development and infection. In several cases the presence of the nucleus near the tip of the cell is necessary for proper growth. Mutants of *Neurospora crassa* and *Aspergillus nidulans* that are affected in nuclear migration are also impaired in hyphal growth (for a review, see Morris 2000). We assume that this holds also true for *P. viticola*, an organism belonging to the oomycetes.

Key events of early development depend on cytoskeleton

The application of drugs that impaired the function of microtubules and of actin microfilaments had dramatic and specific effects on the development of spores of *P. viticola* that allow to identify key events that are dependent on the cytoskeleton and can be connected with the microscopical data (Fig. 2).

Both EPC and latrunculin caused an elevated frequency of encysted spores (Figs. 3B and 4B) even in the absence of salt (Figs. 3C and 4C). However, these encysted spores did not seem to form germ tubes, as the frequency of spores with a germ tube decreased dramatically after treatment with these drugs (Figs. 3E and 4E).

The immunofluorescence data show that during encystation, tubulin is repartitioned into one hemisphere (Fig. 2B) and later into the growing germ tube itself (Fig. 2F). The gradient of tubulin in encysted spores might be related to a role of microtubules in the generation and maintenance of cell polarity as reported for other oomycetes such as *Phytophthora cinnamomi* (Hyde and Hardham 1993) or for other tip-growing cells such as pollen tubes of *Arabidopsis thaliana* (Baskin et al. 1994). The finding that the formation of germ tubes is inhibited by EPC (Fig. 3E) suggests the participation of microtubules in the generation of cell polarity or in the expression of this cell polarity as germ tube growth. The filamentous tubulin structures that are observed along with patches of tubulin during the emergence of the germ tube (Fig. 2F) point to a role of microtubules for germ tube growth. The microtubules observed in the germ tube might be related to the migration of nuclei into the germ tube (Fig. 5C) and the transport of nuclei that proceeds along microtubules in fungal hyphae (for a review, see Morris 2000).

Actin is repartitioned as well, even more extremely, to one pole of the spore (Fig. 2 A) and, subsequently, to the tip of the growing germ tube (Fig. 2 E). Cell polarity has been shown to be established by microfilaments in zygotes of *Fucus* sp. (Quatrano and Shaw 1997) or hyphae of the oomycete *Saprolegnia ferax* (Heath et al. 2000). The accumulation of actin at one spore pole (Fig. 2 A) might therefore represent the site where the germ tube will emerge. To test this hypothesis, it would be required either to visualize actin in living cells or to align germ tube emergence by external factors (such as the attachment structures of a host stoma) and test whether this will result in an alignment of polar actin accumulations.

The developmental dynamics of cytoskeletal patterns require the dissolution of preexisting microtubule and actin structures during the encystation in response to salt. The observed distribution of actin during the individual stages indicates how the dissolution and repolymerization of this structure could work. Actin was found to form granulous plaquelike structures along with filamentous structures in encysted (Fig. 2 A) and germinated spores (Fig. 2 E). These actin plaques seem to be typical for oomycetes (e.g., Heath 1990). For example a stage-specific assembly and disassembly of actin plaques was shown for *Phytophthora cinnamomi* (Jackson and Hardham 1998). Probably, these plaques are organization centers related to the reorganization of cell structure and the transition of the cell into a new stage. The accumulation of actin in one pole of encysted spores (Fig. 2 A) seems to be one of the earliest markers of germ tube formation. During the subsequent stages of germ tube formation, these actin plaques are observed to redistribute from the residual spore to the germ tube, whereas distinct actin caps are observed in the tip of the germ tube (Fig. 2 E). Such actin caps have been described for other oomycetes as well (e.g., Temperli et al. 1990, Harold and Harold 1992). Longitudinal filaments of actin lead from the base of the germ tube to these caps (Fig. 2 E). These filaments could serve as tracks for the actin plaques as well as for specific populations of vesicles, comparable to the vesicles containing cell wall material in the organelle exclusion zone in pollen tubes of higher plants (Miller et al. 1996).

In fact, vesicles are abundant in encysted spores (Fig. 2 C), indicating increased secretory activity. This accumulation might represent a preparatory step for the formation of the germ tube, since this event takes place rapidly (Fig. 1 E). During germ tube formation, the vesicle signal is distributed in a polar fashion (Fig. 2 D) with accumulation in the tip and in bulges of the germ tube, where growth activity is located. The accumulation of vesicles in this region might be related to the transport of cell wall material and enzymes required for the intussusception of wall material at the tip (for reviews, Gow 1995, Heath 1995). The observed colocalization of cytoskeletal elements and vesicles in the tip of the germ tube indicates that vesicles are transported along the cytoskeleton.

The increased spontaneous encystation in response to EPC or latrunculin in the absence of salt (Fig. 3 C and 4 C) indicates that microtubule or actin dissolution per se can trigger the encystation event. However, the reassembly of new microtubule and microfilament structures cannot proceed in the presence of the drug, resulting in the inhibition of germ tube formation. These data thus pinpoint the developmental events that are dependent on microtubules and actin microfilaments: the dissolution of microtubule and microfilament structures in the swarming zoospore seems to be a trigger for encystation, and germ tube formation requires the assembly of new microtubules and actin microfilaments in the encysted spore, either for the establishment of cell polarity that controls the site of germ tube emergence as reported for the oomycete *Phytophthora cinnamomi* (Hyde and Hardham 1993) or, alternatively, for the growth of the germ tube itself.

The present study is confined to the early development culminating in the formation of a germ tube. On the host plant, these steps are followed by the formation of an infection vesicle and penetration of the primary hyphae into the substomatal cavity. This poses the fascinating question, how the germ tube is oriented towards the stomata. Future studies will therefore focus on the factors that are responsible for the efficient localization of the infection vesicle into the substomatal cavity.

References

Arndt-Jovin DJ, Jovin TM (1989) Fluorescence labeling and microscopy of DNA. Methods Cell Biol 30: 417–448

Baskin TI, Wilson JE, Cork A, Williamson RE (1994) Morphology and microtubule organization in Arabidopsis roots exposed to oryzalin and taxol. Plant Cell Physiol 35: 935–942

Cohen Y, Reuveni M, Baider A (1999) Local and systemic activity of BABA (DL-3-aminobutyric acid) against *Plasmopara viticola* in grapevines. Eur J Plant Pathol 105: 351–361

Fujimura M, Kamakura T, Inoue H, Inoue S, Yamaguchi I (1992) Sensitivity of *Neurospora crassa* to benzimidazoles and *N*-phenylcarbamates: effect of amino acid substitutions at position 198 in β-tubulin. Pesticide Biochem Physiol 44: 165–173

Gow NAR (1995) Tip growth and polarity. In: Gow NAR (ed) The growing fungus. Chapman and Hall, London, pp 277–299

Harold RL, Harold FM (1992) Configuration of actin microfilaments during sporangium development in *Achlya bisexualis*: comparison of two staining protocols. Protoplasma 171: 110–116

Heath IB (1995) The cytoskeleton. In: Gow NAR (ed) The growing fungus. Chapman and Hall, London, pp 99–134

– Gupta G, Bai S (2000) Plasma membrane-adjacent actin filaments, but not microtubules, are essential for both polarization and hyphal tip morphogenesis in *Saprolegnia ferax* and *Neurospora crassa*. Fungal Genet Biol 30: 45–62

Hyde GJ, Hardham AR (1993) Microtubules regulate the generation of polarity in zoospores of *Phytophthora cinnamomi*. Eur J Cell Biol 62: 75–85

Jackson SL, Hardham AR (1998) Dynamic rearrangement of the filamentous actin network occurs during zoosporogenesis and encystment in the oomycete *Phytophthora cinnamomi*. Fungal Genet Biol 24: 24–33

Kropf DL, Bisgrove SR, Hable WE (1998) Cytoskeletal control of growth in plant cells. Curr Opin Cell Biol 10: 117–122

Lowry DS, Fisher KE, Roberson RW (1998) Establishment and maintenance of nuclear position during zoospore formation in *Allomyces macrogynus*: roles of the cytoskeleton. Fungal Genet Biol 24: 34–44

Miller DD, Lancelle SA, Hepler PK (1996) Actin microfilaments do not form a dense meshwork in *Lilium longiflorum* pollen tube tips. Protoplasma 195: 123–132

Morris NR (2000) Nuclear migration: from fungi to the mammalian brain. J Cell Biol 148: 1097–1101

Müller K, Sleumer H (1934) Biologische Untersuchungen über die Peronosporakrankheit des Weinstockes mit besonderer Berücksichtigung ihrer Bekämpfung nach der Inkubationskalendermethode. Landwirtschaftl Jahrb Z Wissenschaftl Landwirtschaft 79: 509–576

Nick P (1999) Signals, motors, morphogenesis: the cytoskeleton in plant development. Plant Biol 1: 169–179

Quatrano RS, Shaw SL (1997) Role of the cell wall in the determination of cell polarity and the plane of cell division in Fucus embryos. Trends Plant Sci 2: 15–21

Spector I, Shochet NR, Kashman Y, Groweiss A (1983) Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. Science 219: 493–495

Temperli E, Roos U-P, Hohl HR (1990) Actin and tubulin cytoskeletons in germlings of the oomycete fungus *Phytophthora infestans*. Eur J Cell Biol 53: 75–88

– Shochet NR, Blasberger D, Kashman Y (1989) Latrunculins – novel marine macrolides that disrupt microfilament organization and affect cell growth I: comparison with Cytochalasin D. Cell Motil Cytoskeleton 13: 127–144