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ORIGINAL ARTICLE

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# The host guides morphogenesis and stomatal targeting in the grapevine pathogen *Plasmopara viticola*

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**Abstract** The oomycete grape downy mildew (*Plasmopara viticola* Berk. & Curt. Ex de Bary) is a serious pathogen of grapevine and spreads by extremely efficient cycles of asexual propagation. The high efficiency must involve efficient sensing of the host. We therefore analyzed the time course and morphology of the early development of this pathogen in a host system, by infection of leaf discs of grapevine (*Vitis vinifera* L. cv. Müller-Thurgau), and in a host-free system. Host factors were demonstrated to influence pathogen development in the following ways: (i) the release of zoospores from mature sporangia was accelerated, (ii) the morphogenesis of the germ tube was coordinated, and (iii) the zoospores were targeted to the stomata by factors that depended on stomata closure. The findings show that the early development of *P. viticola* is regulated, specifically and coordinately, by factors originating from the host plant.

**Keywords** Host-pathogen interaction · Oomycete · *Plasmopara* (downy mildew) · Polar cell growth · *Vitis* (pathogen targeting)

**Abbreviations** ABA: abscisic acid · DAPI: 4′,6-diamidino-2-phenylindole · FDA: fluorescein diacetate

## Introduction

The phytopathogenic oomycete downy mildew (*Plasmopara viticola*), originally inhabiting wild species of *Vitis* in North America, was reported for the first time in Europe in 1878 and since then has posed a major problem in vineyards all over the world. At high humidity, it can infect large areas within a short period of time (Müller and Sleumer 1934) and causes huge losses in yield by affecting leaves and young grapes. Sexual propagation exists prior to overwintering of infectious spores, which are of agronomic importance as they provide the first source of inoculum in the year. However, it is the rapid sequence of asexual propagation cycles (sometimes as short as 4 days) that is responsible for the efficient spread of this oomycete during the growing season (Vercesi et al. 1999).

Most of the development takes place within the host leaf, impeding curative approaches to pathogen control. For this reason and with respect to management of fungicide resistance, strategies targeted to the early stages of development appear to be more promising, because then the pathogen is still found on the lower surface of the leaves. Unfortunately, the cellular and molecular events characterizing this early phase of development are still far from being understood, which is mostly due to the fact that, so far, *P. viticola* has turned out to be recalcitrant to any attempt at host-free cultivation.

At high humidity and warm temperatures, the lemon-shaped sporangia release several flagellate zoospores that swarm within the thin water film covering the lower leaf surface (Müller and Sleumer 1934). When the zoospores encounter a stoma, they shed their flagella and encyst. Subsequently, a germ tube emerges from each spore and reaches into the substomatal cavity, where it dilates into an infection vesicle. From this infection vesicle, a primary hypha emerges (opposite to the site of the spore), and develops a mycelium that spreads within the leaf tissue, extending mainly into the intercellular space of the spongy parenchyma and forming haustoria that penetrate into the cells of the host.

Recently, a host-free system has been established that allows the early development of *P. viticola* to be studied from zoospore release until the formation of a germ tube

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(Riemann et al. 2002). Using this system, the time courses of zoospore release, encystment, and germ-tube formation could be characterized on a quantitative level to assess the role of the cytoskeleton in early development. Whereas the formation of a germ tube was dependent on both actin microfilaments and microtubules, the release of zoospores from the mature sporangium was found to be independent of these factors.

In order to infect the host successfully, the released zoospores have to meet two requirements: (i) they must be targeted to the stomata and (ii) they must orient cell polarity such that the germ tube penetrates into the substomatal cavity. In other oomycete pathogens, the attraction and subsequent development of zoospores are often controlled by factors released from the host, such as amino acids, pectins (*Halophytophthora vesicula*: Leano et al. 1998) or phenolic derivatives (*Phytophthora sojae*: Morris and Ward 1992; Connolly et al. 1999; *Phytophthora cinnamomi*: Cahill and Hardham 1994). These host factors do not seem to be very specific. However, specific host substances have been reported as well (*Aphanomyces cochlioides*: Takayama et al. 1998). As an alternative attractant, electrical fields have been discussed extensively (Morris et al. 1992; Morris and Gow 1993; Cahill and Hardham 1994), but were later shown to be dispensable, since zoospore taxis remains functional when the pH is buffered (Morris et al. 1995). It is also possible that guiding factors originating from the host are amplified by attractants from other zoospores that have already successfully tracked the target cell. This so called adelphotaxis has been reported for the oomycete *Achlya* (Thomas and Peterson 1990). In most oomycetes, the polarity of the zoospores seems to be present, but during the attachment to the host cell (so-called docking) it is reoriented such that the prospective germ-tube pole is aligned correctly (Deacon 1996).

It is difficult to study, in a host-free system, events such as the targeting to the host cell or the orientation of cell polarity, as long as the signals that guide the zoospores remain unknown. In order to identify such signals, it has first to be demonstrated that they exist. We therefore compare in the present work the early development of *P. viticola* in the host-free system with the ''natural'' situation on the host leaf. We can demonstrate that host factors promote and control the early development, especially the release of zoospores from the sporangia, and the morphogenesis of the germ tube. We demonstrate further that the zoospores find the stomata actively and are guided by host factors that are released from open stomata.

## Materials and methods

### Pathogen material

*Plasmopara viticola* (Berk. & Curtis) Berl. & DeToni was cultivated in the greenhouse on the host plant. To infect of the plants, the lower surface of the leaves was sprayed with an aqueous suspension of sporangia, by means of a chromatography sprayer (Ecospray; Roth, Karlsruhe, Germany), until run-off. A plastic bag was rinsed with water, and the entire plant covered with the bag overnight. After 4–7 days of incubation in the greenhouse, sporulation could be triggered by covering the pre-infected plant overnight with a humid plastic bag. Successful infection became manifest as a white, powdery coating on the lower leaf surface. For the physiological experiments, only freshly harvested material was utilized.

### Plant material

Scions of the susceptible grapevine (*Vitis vinifera* L.) cultivar Müller-Thurgau were raised in a greenhouse in pots on soil under a 16 h:8 h light:dark cycle using artificial daylight [400 W, Vialox NAV-T (SON-T); Osram, Mühlheim, Germany] in addition to natural light. The sixth unfolded leaf (counted from the apex) was excised, and its surface sterilized with ethanol, rinsed with distilled water and carefully dried with tissue. Leaf discs of 14 mm diameter were obtained using a cork-borer on an elastic rubber pad. The leaf discs were excised such that they did not contain major veins and were placed top-down on water agar (0.8% w/v). For the experiments with abscisic acid (ABA), the plants were first watered and placed in the dark for several hours to induce stomatal closure and full turgescence of the leaves. Subsequently, they were returned to the light at 22 °C. The sixth unfolded leaf (counted from the apex) was excised at the base of the petiole, and the cut surface trimmed with a razor blade such that the edge was cut obliquely in order to ensure maximal water uptake. The petioles were then placed in water or different concentrations of ABA, and the leaves incubated for 3 h in the light. Leaf discs were obtained from these leaves as described above. It should be noted that the leaf discs did not have any direct contact with the ABA.

### Inoculation

The sporangia were resuspended in water and adjusted to 40,000 sporangia per ml using a hemacytometer (Fuchs-Rosenthal, Thoma, Freiburg). 100 µl of this suspension was inoculated per leaf disc corresponding to 4,000 sporangia per disc. For the experiments on stomatal targeting, the density was doubled to 80,000 sporangia per ml.

### Induction of pathogen development in the host-free system

Sporangia of *P. viticola* were suspended to homogeneity in 10 ml distilled water and transferred into a petri dish. After 3 h of incubation in white light (5 $W \cdot m^{-2}$) in a phytotron (Type RTL 4; Ehret, Emmendingen, 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. Encystment occurred virtually instantaneously upon addition of sodium chloride, and 1 h later numerous spores had generated a germ tube. The frequency of different stages was scored under a light microscope in aliquots of 50 µl using a hemacytometer (Fuchs-Rosenthal). The curves for the host-free system represent the data from at least 4 individual time-course experiments comprising more than 17,000 individual spores.

### Visualization of the early development on leaf discs

The early stages (sporangia, zoospores, encysted zoospores and young germ tubes) could be visualized using fluorescein-diacetate [FDA (Sigma-Aldrich); 1:1000 dilution from a stock solution (0.5% w/v in acetone) in 0.1% v/v Tween-20] added directly to the inoculum. The attachment of zoospores to the stomata was best visualized using Blankophor (Bayer, Leverkusen, Germany; 0.1% w/v in 10% ethanol) added directly to the inoculum after mild depigmentation (30 min in 9.2 mM trichloroacetic acid in 20% v/v

chloroform and 80% v/v ethanol). The subsequent stages, especially the infection vesicle and the primary hyphae, but also the mycelium and haustoria, could be visualized with 0.05% w/v aniline blue (Sigma-Aldrich) in 0.067 M $K_2HPO_4$ (pH 9.8) for 5 min after rigid depigmentation of the host tissue (autoclaving for 10 min at 121 °C and 1.2 bar in 1 M KOH). For all three dyes, the fluorescence was analyzed on the leaf disc using excitation at 395–440 nm, a beam splitter at 460 nm and a 470-nm low-pass barrier filter on an epifluorescence microscope (Axiophot; Zeiss, Göttingen, Germany).

### Quantification of early development

The relative abundance of each developmental stage was determined in relation to the maximal occurrence of the respective stage. The time courses were repeated three times, using two to three leaf discs per time point. For the quantification of stomatal targeting, the average number of spores per stoma was determined in 2 independent time series, scoring 2 leaf discs per time point and 1,536 individual spores. The dose-response curve for the influence of ABA on stomatal targeting represents the data from 2 independent time series, scoring 4 leaf discs per time point and 4,000 stomata with attached spores.

## Results

### Establishment of protocols to visualize early development of *P. viticola* on leaf discs

None of the fluorescent dyes tested was able to visualize all pathogen structures (data not shown). To observe development in the presence of the host, it was therefore necessary to adapt visualization and depigmentation (to overcome chlorophyll fluorescence) to the developmental stage under observation. The advanced stages required and tolerated rigid depigmentation (autoclaving the leaf discs in KOH), and were best stained with aniline blue. For the early stages the rigid depigmentation displaced the pathogen from the leaf surface (data not shown). As these stages can be observed on the surface of the leaf, reducing the demand for complete depigmentation, a milder protocol based on chloroform, ethanol and trichloroacetic acid was successfully employed and combined with staining by FDA or (for the encysted zoospores) Blankophor.

### The release of zoospores is accelerated in presence of the host

The time course of zoospore release from the sporangia was followed after inoculation with a sporangial suspension on leaf discs of grapevine using FDA for visualization (Fig. 1a). Free zoospores appeared from 40 min after inoculation and were most abundant about 90 min after inoculation. At this time, sporangia that still contained zoospores had become very rare. After 90 min, the frequency of free zoospores dropped progressively, accompanied by the appearance of the subsequent stages such as encystment and germ-tube formation (data not shown). Compared with the time course in a host-free system (Fig. 1a; according to Riemann et al. 2002 based on 14,000 individual stages,

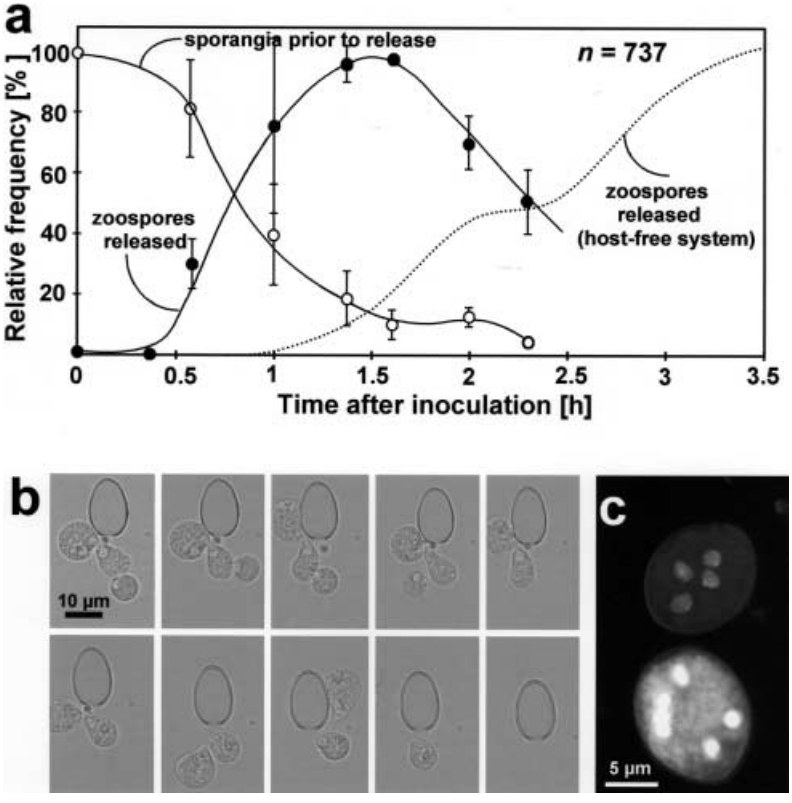



**Fig. 1a–c** Release of zoospores from mature sporangia of *Plasmopara viticola*. **a** Time course of zoospore release after inoculation of leaf discs (*solid curves*) compared with the host-free system (*dotted curve*; according to Riemann et al. 2002 , and based on 14,000 individual stages, standard errors for this curve are in the range of 10% of relative abundance). Abundancies of the respective stages relative to the maximal abundance of each stage are plotted against the time after inoculation. The data are constructed from three independent experimental series each comprising the data of two leaf discs per time point. **b** Time-lapse series of the final phase of zoospore release in the host-free system. The images cover an interval of a few seconds. **c** Visualization of nuclei by DAPI in mature sporangia prior to inoculation

standard errors for this curve are in the range of 10% of relative abundance), the release of zoospores on the leaf discs was accelerated by about 1–2 h. The release itself was observed to be a rapid process, taking place within a few minutes irrespective of the system used (Fig. 1b). However, the lag time between inoculation and release was much shorter when the sporangia were in contact with the host. One sporangium can release several, usually three to six, zoospores (Fig. 1b).

To test whether the lag time between inoculation and release was related to nuclear divisions, mature sporangia were stained with 4′,6-diamidino-2-phenylindole (DAPI). Four to six nuclei for each sporangium were already observed prior to inoculation (Fig. 1c). Thus, there were no indications of nuclear divisions during the lag time between inoculation and zoospore release.

## The morphogenesis of germ tubes and infection vesicles is more regular in the presence of the host

When the zoospores attach to the stoma, they shed their flagella, encyst and develop a germ tube reaching into the substomatal cavity (Fig. 2d). Subsequently, the nucleus and the complete cytoplasm translocate from the spore into the apical half of the germ tube. When the residual spore is empty, a septum is formed in the apical part of the germ tube, and the apex of the germ tube begins to swell until a so-called infection vesicle develops (Fig. 2d). At the apical pole of this infection vesicle, which is oriented into the substomatal cavity, tip growth resumes and a primary hypha emerges (Fig. 2d) that will later prime the mycelium. When the appearance of infection vesicles with primary hyphae was scored over time after inoculation of leaf discs (Fig. 2a), they could be observed from about 1 h after inoculation, reaching a maximum value at 2 h after inoculation. Compared with the data for zoospore release (Fig. 1a, Fig. 2a, dashed curve), infection vesicles and primary hyphae appear on a parallel time course, but shifted by about 40 min. This indicates that the development from zoospore release to encystment, and formation of a germ tube till the development of infection vesicles and primary hyphae is a relatively rapid process that is completed within 40 min.

The formation of a germ tube (Riemann et al. 2002) and, occasionally, the development of subsequent stages can be induced by addition of sodium chloride. However, morphogenesis in the host-free system appears to be more susceptible to stochastic deviations. Whereas branching and bulging of germ tubes is typical in the host-free system (Fig. 2b), a zoospore that develops for the same period on the host will form one unbranched, straight germ tube (Fig. 2c). Changes in morphogenesis can also be detected by morphometric parameters. For instance, the length of the germ tube between the point where it emerges from the spore and the septum (Fig. 2d) is highly constant for spores that germinate on the host ($159 \pm 0.6$, $n = 20$), whereas it is increased and more variable for germination in the host-free system

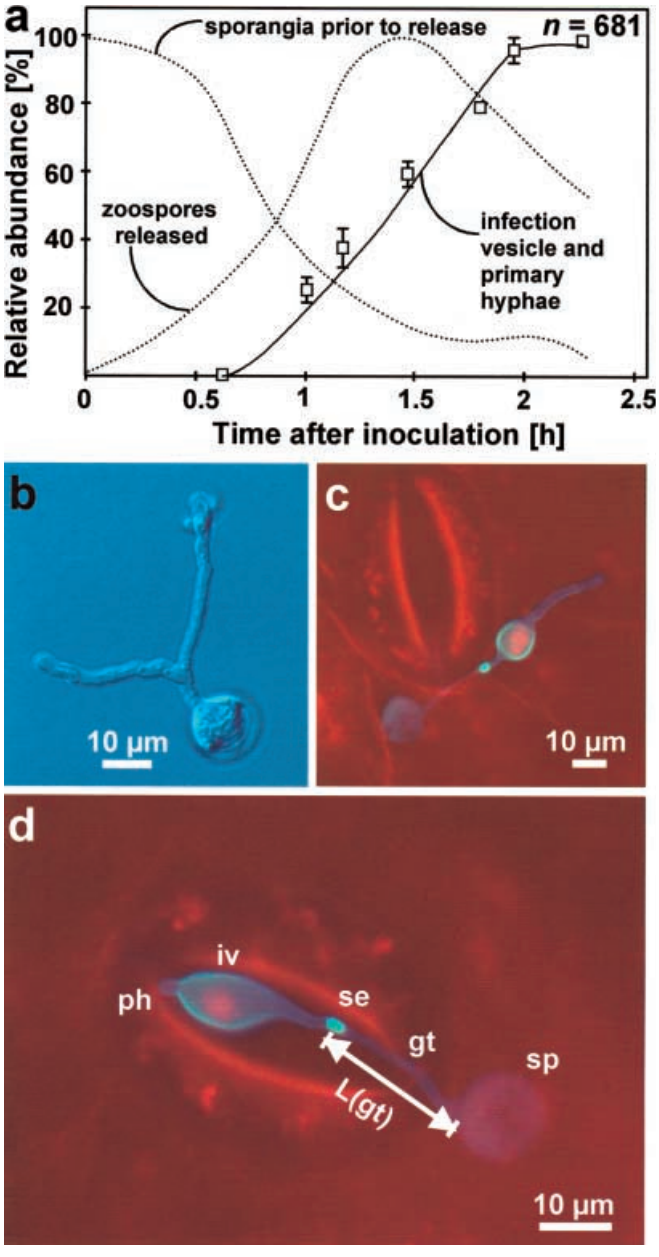



**Fig. 2a–d** Formation of germ tubes, infection vesicles and primary hyphae by *P viticola*. **a** Time course for the formation of infection vesicles with primary hyphae after inoculation of leaf discs (*solid curve*) in comparison with the time course of zoospore release (*dotted curves*, see Fig. 1a). The abundancies of the respective stages relative to the maximal abundance of each stage are plotted against the time after inoculation. The data are constructed from three independent experimental series, each time comprising the data for two leaf discs per time point. **b** Bright-field image of a zoospore forming a branched germ tube in the host-free system. **c** Zoospore of comparable developmental age with a straight, unbranched germ tube, infection vesicle and primary hypha developed on the host, as visualized by aniline blue **d** Definition of the germ-tube length [*L(gt)*], as an indicator of morphogenetic order, as the distance between the first septum (*se*) and the point of emergence from the spore (*sp*) *gt* Germ tube, *iv* infection vesicle, *ph* primary hypha

($159 \pm 0.6$ μm versus $232 \pm 16$ μm, $n = 20$, $P = 0.00018$). These observations indicate that the presence of the host synchronizes and coordinates the development of the germ tube.

### The zoospores are targeted to the stoma by host factors depending on stomatal opening

To follow the time course of stomatal targeting (Fig. 3a), zoospores were released in the absence of the host and only inoculated on leaf discs after complete release. After variable time intervals, encysted zoospores were then visualized by Blankophor and scored. A lag time of 5 min was required until the first stomata occupied by encysted zoospores could be observed (Fig. 3a). However, 8 min after inoculation most stomata were already occupied by one or two encysted spores (Fig. 3c, d), and around 20 min after inoculation a plateau was reached with about four zoospores per stoma (Fig. 3a, e). Interestingly, from 35 min after inoculation, the curve increased in a second wave to a plateau of about six zoospores per stoma at 45 min after inoculation. In contrast to this aggregation of encysted zoospores at the stomata, the zoospores encysted in isolation in the host-free system.

To test the possibility that the zoospores are targeted by host factors that are released from open stomata, leaf discs were obtained from leaves that had been pre-incubated on increasing concentrations of ABA. This treatment resulted in a dose-dependent increasing proportion of closed stomata (data not shown). The ABA dose–response of the final number of zoospores per stoma (measured 90 min after infection; Fig. 3b) reveals a progressive inhibition of stomatal targeting for concentrations exceeding 3 μM ABA, with an inhibition by about 40% of the control value at 100 μM ABA.

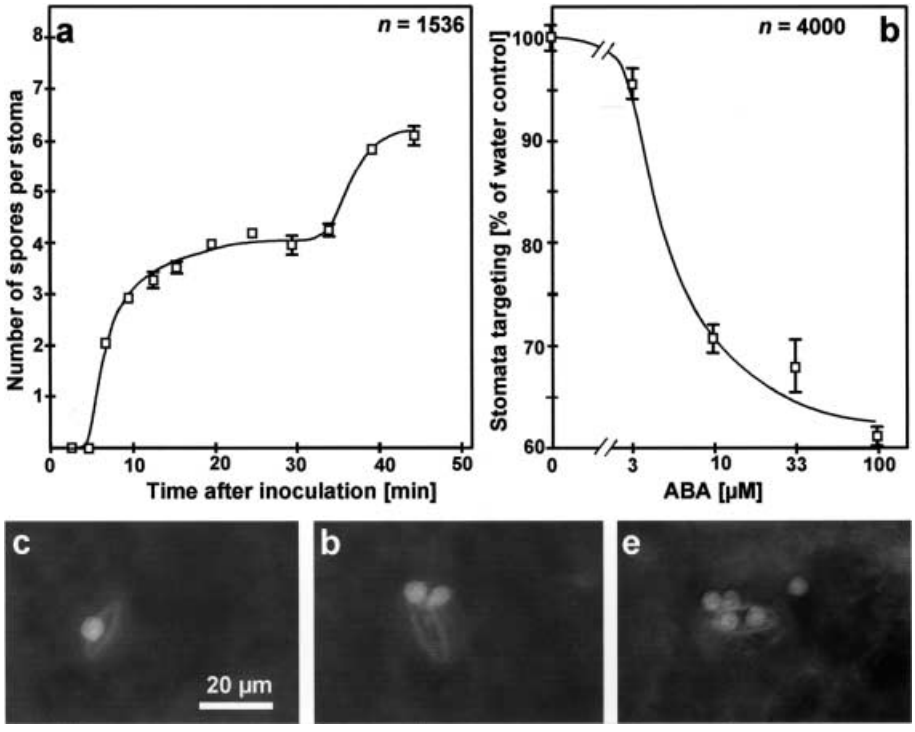
These data show that the released zoospores are rapidly targeted to the stomata (Fig. 3a), and that they are guided by host factors that can be inhibited by closing the stomata via ABA (Fig. 3b).

**Fig. 3a–e** Targeting of zoospores to the stomata in *P viticola*. **a** Time course for the targeting. The average number of zoospores per individual stoma is plotted against the time after inoculation. In this experiment, the zoospores had been completely released in the cell-free system prior to inoculation. The data are constructed from two independent experimental series, each time comprising the data from two leaf discs per sample. **b** Dose–response relation for the ABA-inhibited targeting of stomata by zoospores. The host leaves were pre-incubated with the respective concentration of ABA administered via the petioles, and the zoospores inoculated onto leaf discs obtained from these leaves. The data are constructed from two independent experimental series, each time comprising the data from 4 leaf discs per sample corresponding to 4,000 stomata. **c–e** Zoospores encysted at the stomata as visualized by Blankophor 8 min (**c, d**), and 25 min (**e**) after inoculation

## Discussion

### The early development of *P. viticola* is controlled by host factors

The early development of *P. viticola* was analyzed on leaf discs and compared with the development triggered in a host-free system (Riemann et al. 2002). These investigations revealed two key events that differed when they took place on the host rather than in the host-free system:



1. The release of the zoospores from the mature sporangia was accelerated on the surface of the leaf by about 1–2 h compared with the host-free system (Fig. 1a). Host factors therefore stimulate either the release of zoospores from the sporangium or events that precede this release. The release of the zoospore itself seems to be a fast process that even in the host-free system occurs within a few seconds (Fig. 1b) and thus cannot be responsible for the long lag-phase observed in the host-free system. Thus, essential events preceding the zoospore release in sensu strictu are accelerated by the presence of the host. These seem to be fairly slow events – even on the leaf disc the release of zoospores requires about 90 min. This is surprising, considering the extreme velocity of the subsequent development to the appearance of primary hyphae (Fig. 2a). Since four to six nuclei are already present in each sporangium prior to inoculation (Fig. 1c), consistent with the number of released zoospores (see, e.g. Fig. 1b), there is no indication that the long lag-phase is caused by nuclear divisions. However, preliminary electron-microscopical studies of mature sporangia indicate that the nuclei are not separated by plasma membranes, consistent with findings in other oomycetes such as *Phytophthora cinnamomi* (Hyde and Hardham 1993) and *Allomyces macrogynus* (Fisher et al. 2000). The sporangium therefore represents a syncytium, and during the period prior to zoospore release the individual zoospores have to be separated from each other. This requires extensive synthesis of membranes and vesicle flow towards the sites where the individual spores are separated. In addition, the zoospores have to form flagella. Both represent time-consuming events and thus plausible targets for the accelerating effect of host factors. The host might either stimulate the secretory activity within the sporangium or provide spatial cues that guide this activity to sites where the formation of separating membranes is primed.
2. The host appeared to coordinate the morphogenesis of germ tubes and primary vesicles (Fig. 2b, c). Branching and bulging of the germ tube, which was common in the host-free system (Fig. 2b), was suppressed on the host (Fig. 2c). Moreover, in the host the swelling of the germ tube into an infection vesicle and the septa formed at the basal end of the vesicle were precisely tuned with respect to the growth of the germ tube, whereas they occurred later and less synchronously in the host-free system. The host might act to stabilize the polarity of the growing germ tube and thus ensure that only one germ tube is produced. In this context it would be interesting to search for factors responsible for the so-called docking known from other phytopathogenic oomycetes. These host factors seem to reorient the polarity of the zoospore during the attachment to the host cell such that the prospective germ-tube pole is aligned correctly (Deacon 1996).

The zoospores are targeted by host factors that are released from open stomata. The targeting of the zoospores to the stomata and the encystment at the stomata occur within a few minutes (Fig. 3a). Principally, targeting to the stomata could be either achieved by active taxis or passively by accidental trapping of randomly moving zoospores at the stomata. The very short lag time (less than 10 min) and the rapid saturation of the process (within 40 min) militate against a random movement. Moreover, the residual number of zoospores that have not attached to stomata is extremely low (data not shown), which argues strongly for active taxis. The factor responsible for this taxis remains unknown. However, it is possible to impair the action of this factor in a dose-dependent manner by using ABA to close the stomata (Fig. 3b). This suggests that the guiding factor is released from the open stomata. It should be emphasized that the impaired targeting of zoospores is not caused by effects of ABA on the mobility or tactic behaviour of the zoospores, although this has been described earlier (Cahill and Hardham 1994). In the present study, the hormone was deliberately administered to the leaf via the petiole such that it was not in contact with the zoospores. The inhibition of stomatal targeting (Fig. 3b) must therefore be caused by the closure of stomata induced by ABA. The most straightforward interpretation of these results is to assume that the spores are targeted by chemotaxis dependent on a gradient of volatile or easily soluble substances released from the substomatal cavity. However, other signals such as electrical fields (Morris et al. 1992; Morris and Gow 1993; Cahill and Hardham 1994) released from the stomata or mechanical cues (Hoch et al. 1987; Kwon et al. 1991; Zhou et al. 1991) that depend on stomatal aperture should be considered as well. Whether primary cues originating from the host could subsequently be amplified by signals released from other spores that have already successfully tracked the target cell, similar to the adelphotaxis described for the oomycete *Achlya* (Thomas and Peterson 1990) is difficult to judge – however, the encystment in the host-free system is not accompanied by aggregation of mobile spores.

## Outlook

The comparison between early development in the host-free system versus inoculation of leaf discs uncovered three key events by which host factors regulate pathogen development. (i) The release of zoospores from mature sporangia is accelerated. Therefore, the formation of membranes and flagella prior to zoospore should be followed by specific fluorescence dyes and electron microscopy. (ii) The morphogenesis of the germ tube is coordinated during encystment. Therefore, the spatial relationship between the basal body of the flagella, nucleus and the point of germ-tube emergence should be analyzed by electron microscopy during docking and encystment. (iii) The zoospores are targeted to the

stomata by factors that depend on stomatal closure. Different candidate substances such as amino acids, isoflavones or pectins, but also cell-wall fragments of the host leaf, should be tested for their capacity to act as chemotactic attractants. It would also be worth analyzing the targeting process on leaf discs through a taxonomic cline of wild *Vitis* and *Cissus* species to define the specificity of the attractant. In the long term, a deeper understanding of the interaction between host factors and pathogen development will necessitate the establishment of a molecular biological profile for *P. viticola*. This should allow the identification of early genes that are induced in the syncytium prior to zoospore formation and release, and the genes that are induced during the docking process at the stoma. These genes should provide primary targets for the preventive control of this phytopathogen.

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