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Arabidopsis Is Susceptible to Infection by a Downy Mildew Fungus

Eckhard Koch and Alan Slusarenko[1]

Institut für Pflanzenbiologie, Zollikerstrasse 107, CH 8008 Zürich, Switzerland

A population of *Arabidopsis thaliana* growing locally in a suburb of Zürich called Weiningen was observed to be infected with downy mildew. Plants were collected and the progress of infection was investigated in artificial inoculations in the laboratory. The plants proved to be highly susceptible, and pronounced intercellular mycelial growth, haustoria formation, conidiophore production, and sporulation of the causal organism *Peronospora parasitica* were all observed. The formation of oogonia, antheridia, and oospores also occurred. In contrast, *Arabidopsis* strain RLD was resistant to infection and none of the above structures was formed. The fungus was localized very soon after penetration of RLD leaf cells, which responded with a typical hypersensitive reaction. The differential interaction of an isolate of *P. parasitica* with two strains of *Arabidopsis* opens up the possibility of cloning resistance determinants from a host that is very amenable to genetic and molecular analysis.

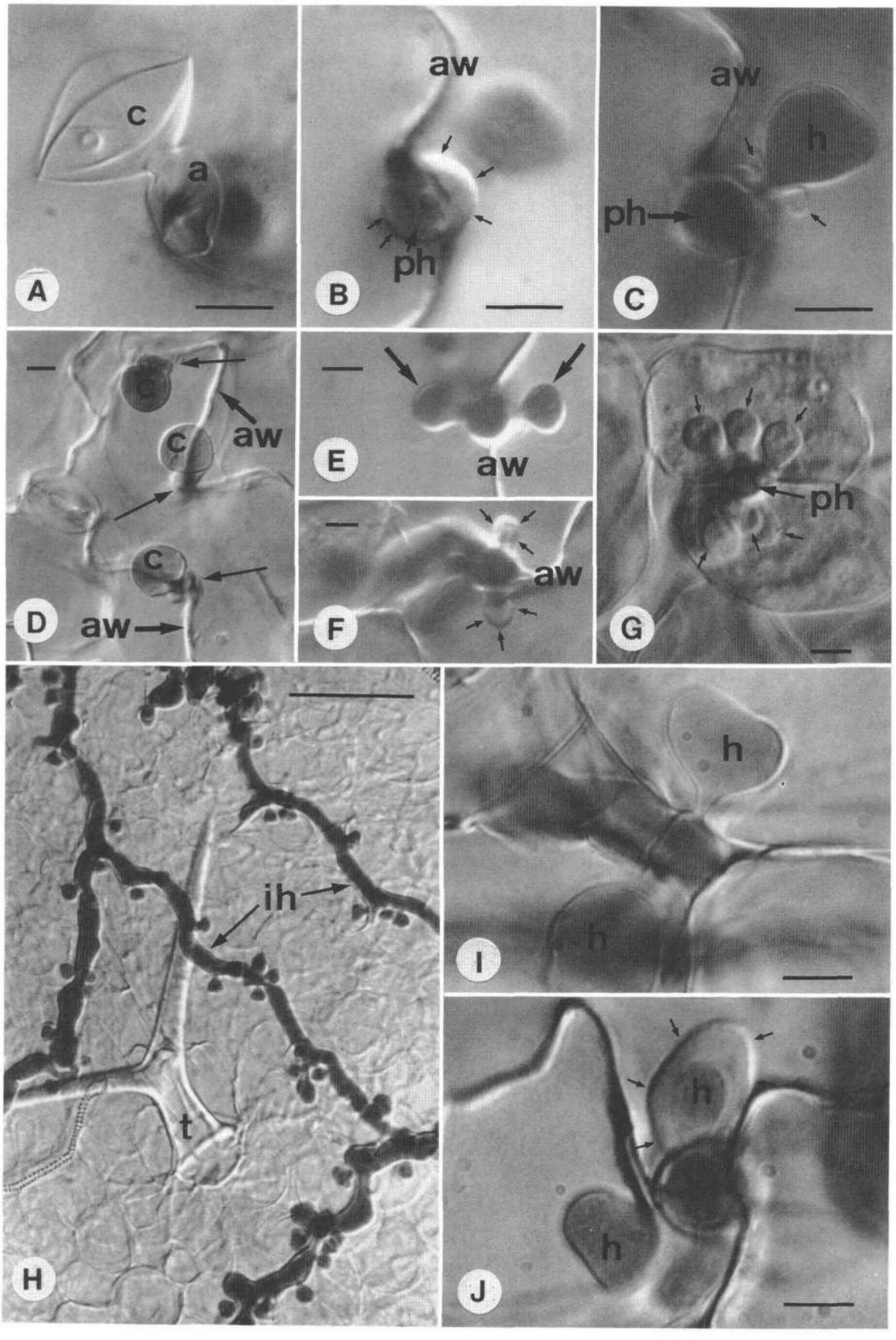
INTRODUCTION

In recent years, the crucifer *Arabidopsis thaliana* has received considerable attention from plant biologists. Because of several attributes, *Arabidopsis* has become a model plant for genetic and molecular biological studies. The small genome, the near-absence of interspersed repetitive DNA, and the availability of parallel genetic maps of marker genes and DNA fragments (Chang et al., 1988; Nam et al., 1989) make gene cloning by chromosome walking particularly attractive in *Arabidopsis* (Meyerowitz, 1989). Investigations of the molecular biology of disease resistance in *A. thaliana* have been hindered by the absence of a suitable pathogen. Indeed, the belief that there are no well-characterized pathogens of *Arabidopsis* is widespread although several promising interactions with plant pathogenic bacteria have been described recently (Somerville, 1989). Davis and Ausubel (1989) circumvented this problem by using a nonspecific elicitor to study potential defense responses in *Arabidopsis*.

Peronospora parasitica (Pers. ex Fr.) Fr. causes downy mildew in members of the Cruciferae. It is widespread in those regions where members of the Cruciferae, particularly brassicaceous crops, are grown. The disease is most prevalent in cool, damp conditions, which favor spore production and dissemination (Channon, 1981). Lindau (1901) mentions *P. parasitica* and *Cystopus candidus* (syn. *Albugo candida*, which causes "white rust" of crucifers) as fungal pathogens of *Stenophragma thalianum* (L.) Cel. [syn. *Arabidopsis thaliana* (L.) Heynh.]. Downy mildews occurring on cruciferous hosts were ascribed to *P. parasitica* until the species was divided up by Gäumann (1918). Based on conidial measurements and cross inoculations, Gäumann recognized 52 species of *Peronospora*, among these *P. arabidopsidis*. Downy mildew on *Arabidopsis* had apparently already been recognized at this time because Gäumann states that his *P. arabidopsidis* was synonymous with "*P. parasitica* Pers. f. *Sisymbrii thaliani* Schneider 1865 (nom. nud. in sched.)." *A. thaliana* (L.) Heynh. and *Sisymbrium thalianum* (L.) Gay are synonymous. Yerkes and Shaw (1959) pointed out the remarkable morphological similarities of the *Peronospora* species that attack crucifers. The current situation is that all isolates of downy mildews infecting members of the Cruciferae are ascribed to *P. parasitica*.

Certainly, based on these and other more recent reports (Brandenburger, 1985), the view that *Arabidopsis* has no pathogens needs reappraisal. Indeed, other fungal pathogens of *A. thaliana* have been isolated recently, e.g., *Rhizoctonia solani* and *Botrytis cinerea* (E. Koch and A. Slusarenko, manuscript in preparation). Pathogens from other cruciferous species have also been shown to be capable of infecting *Arabidopsis*, e.g., *Plasmodiophora brassicae* (E. Koch and P.H. Williams, manuscript in preparation) and *Erysiphe cruciferarum* (E. Koch and A. Slusarenko, manuscript in preparation). There are no reports of the cloning of classical host resistance genes (Somerville, 1989). A major problem in identifying resistance genes is the large size of the plant genome. The differential interaction that we report here, of an isolate of *P. parasitica* with two different strains of *Arabidopsis*, opens up the possibility of cloning resistance determinants from a host that is very amenable to genetic and molecular analysis.

[1] To whom correspondence should be addressed.



RESULTS

Infection Process

Light microscopic observations of whole-leaf mounts 18 hr after inoculation of *Arabidopsis* strain Weiningen revealed that development of the fungus had advanced beyond the initial stages of infection, i.e., haustoria were already present. The majority of conidia were deposited over, or in close proximity to, anticlinal walls of adjoining epidermal cells. Conidia had germinated and formed appressoria, as shown in Figure 1A. Appressoria were, in most cases, produced directly from conidia; however, well-developed germ tubes several spore diameters in length were also observed. Conidia and appressoria were empty by this time (Figure 1A), indicating that the cytoplasm had moved into penetration hyphae that extended from the appressorium and penetrated between the anticlinal walls of adjoining epidermal cells (Figures 1A to 1D). Material apparently deposited by the host in response to the ingress of the pathogen was observed in epidermal cells adjacent to the site of penetration (Figure 1B). Necrosis of epidermal cells in contact with the penetrating hypha was not observed in the Weiningen strain of *Arabidopsis*. Often the first haustorium was inserted into one of the adjoining epidermal cells (Figure 1C). One haustorium in each of the epidermal cells neighboring the penetrating hypha was also observed frequently (Figure 1E). In a few instances, haustorial bodies in epidermal cells were not fully expanded but were encapsulated with a material that had apparently been deposited by the host as a defense mechanism (Figure 1F). Growth of the penetrating hypha was, however, unaffected, and normal haustoria were present in the underlying mesophyll cells. This phenomenon may be related to observations by Chou (1970), who concluded from ultrastructural studies that epidermal cells of cabbage responded much more vigorously to infection with *P. parasitica* than did mesophyll cells. Sometimes the hypha passed between anticlinal walls without forming haustoria in the epidermis. In this case, single or multiple haustoria were produced upon contact of the penetrating hypha with cells of the mesophyll (Figure 1G). Three days after inoculation, extensive growth of the coenocytic, intercellular mycelium was evident on *Arabidopsis* strain Weiningen (Figure 1H). Hyphal strands were multiply branched and haustoria were present in abundance. Necrosis of host cells or signs of retarded growth of the fungus were absent, indicating a high degree of compatibility between host and parasite. Young haustoria were pyriform (Figures 1E and 1G) and later became somewhat heartshaped (Figures 1H and 1I) to multiply lobed. Collar-like structures were often present around haustorial necks. At the time of sporulation, several haustoria were observed that were encased by host cell material. The encasements appeared as thick capsules surrounding the haustorial neck and body (Figure 1J). Encased haustoria had generally reached almost full size. Apparently, this host cell response occurred slowly and was possibly related to the aging of the haustoria.

Observation of tissues of *Arabidopsis* strain RLD 18 hr after inoculation showed that one or both of the epidermal cells adjacent to the penetration hypha had reacted hypersensitively to the pathogen (Figures 2A and 2B). A hypersensitive response (HR), i.e., a rapid, localized necrosis of host cells, indicates a high degree of incompatibility between the host and pathogen. However, the fungus was able to penetrate through to the mesophyll where, although in a few cases branching was observed, apical growth of the hypha quickly ceased. Near the growing tip, the diameter of the hypha was markedly reduced compared with its size in the initial stages of ingress (Figures 2C and 2D). At 48 hr after inoculation, approximately three to five deep blue-staining mesophyll cells were present below penetration sites and adjacent to hyphae. Although the stain penetrates fungal cells quite easily, it is usually excluded

Figure 1. Infection Process and Vegetative Growth of *P. parasitica* in *A. thaliana* Strain Weiningen.

All samples were taken at 18 hr after inoculation unless otherwise stated.

(A) A germinated conidium (c) with an appressorium (a) on the leaf surface; both structures are devoid of cytoplasm. Bar = 10 μm.

(B) A penetration hypha (ph) at the point of entry between anticlinal walls (aw) of two epidermal cells. Note the apposition of material adjacent to the site of penetration (arrows). Bar = 10 μm.

(C) The same infection site as in **(B)** but focused through to the epidermal cells. The penetration hypha (ph) has expanded and the first haustorium (h) has been formed in one of the epidermal cells. Note the material localized at the haustorial neck (arrows). aw, anticlinal walls. Bar = 10 μm.

(D) Germinated conidia (c) on the leaf surface. In the cases shown, the appressoria (arrows) were produced directly from conidia without the formation of a germ tube. Appressoria are positioned over anticlinal walls (aw). Bar = 10 μm.

(E) Simultaneous formation of haustoria (arrows) in both epidermal cells. aw, anticlinal walls. Bar = 10 μm.

(F) Encasements (arrows) in epidermal cells surrounding haustorial initials. aw, anticlinal walls. Bar = 10 μm.

(G) Formation of multiple haustoria (arrows) in mesophyll cells. The penetration hypha (ph) is arrowed. Bar = 10 μm.

(H) Branched intercellular hyphae (ih) with numerous haustoria in mesophyll cells. A trichome (t) on the leaf surface can be seen clearly. Sample taken 3 days after inoculation. Bar = 100 μm.

(I) Fully expanded haustoria (h) in mesophyll cells. Sample taken 3 days after inoculation. Bar = 10 μm.

(J) Normal and encased (arrows) haustoria (h). The encasement is deposited around the haustorial neck and body. Sample taken 7 days after inoculation. Bar = 10 μm.

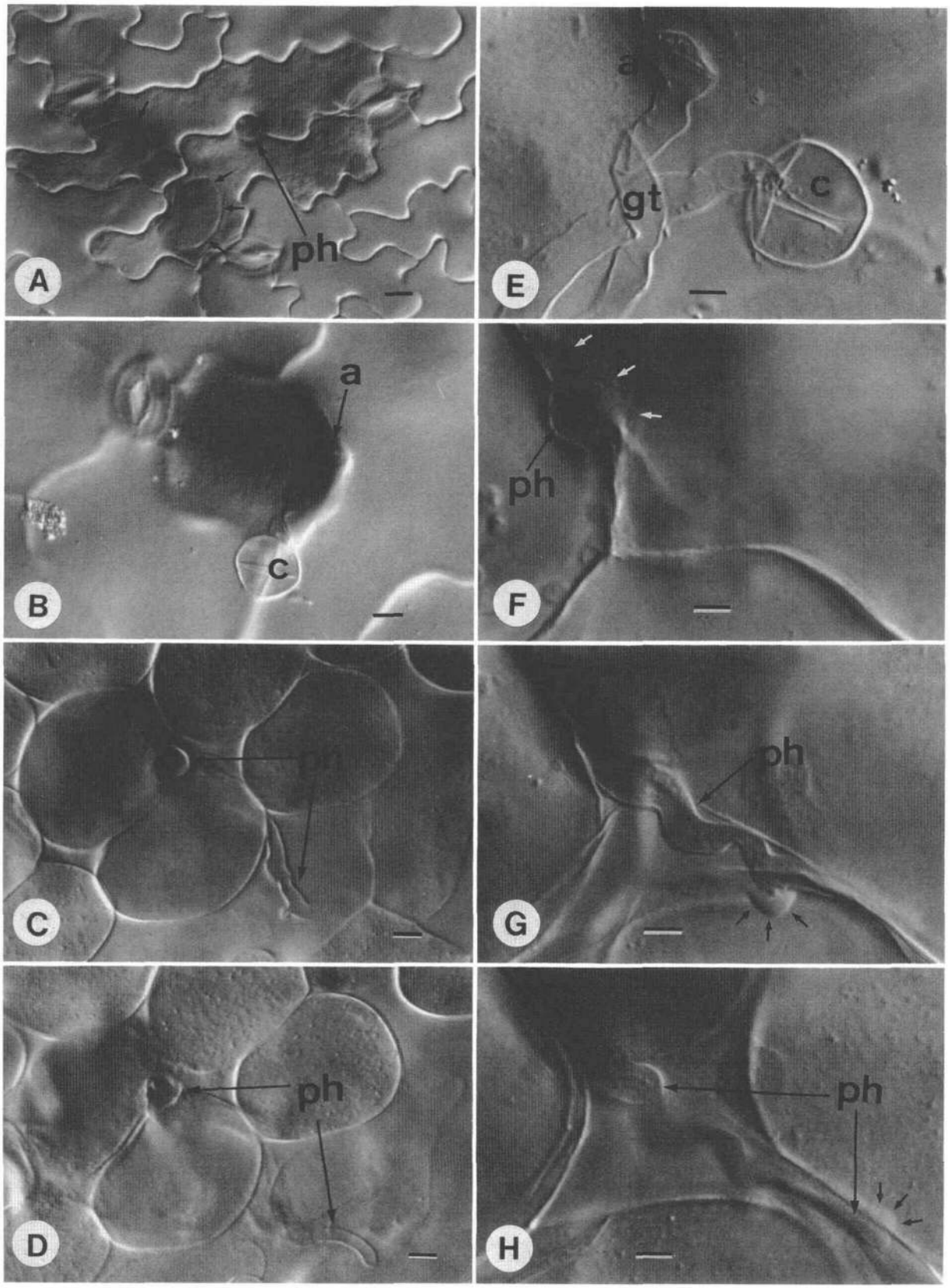


Figure 2. Abortive Infection of *A. thaliana* Strain RLD by *P. parasitica*.

All samples were taken at 18 hr after inoculation.

from healthy plant cells: this is the basis of the differential staining technique used in plant pathology to highlight fungal growth in plant tissues. Penetration of the stain into host cells is indicative of membrane damage. The stain has been used to highlight hypersensitivity responding host cells in incompatible interactions of plants with fungi (Keogh et al., 1980) and bacteria (Slusarenko and Longland, 1986).

Apart from the pronounced HR in strain RLD, the initial stages of infection, i.e., germination of conidia, formation of appressoria (Figures 2B and 2E), and penetration of hyphae between anticlinal walls of epidermal cells (Figures 2A and 2F), all occurred in the same manner as described for infection of *Arabidopsis* strain Weiningen. Figures 2F and 2B show conidial germination and appressorium formation with and without germ tube production, respectively. In addition to the HR, apposition of host material in mesophyll cells at the point of contact with the penetrating hypha was observed (Figures 2G and 2H). Occasionally hyphae in contact with host cells formed a knob-like outgrowth, possibly in an attempt to produce haustoria. Fully developed haustoria, however, were never observed. In samples stained 48 hr after inoculation, intercellular hyphae were very rarely seen. They appeared thin, devoid of cytoplasm, and had in no case advanced beyond the immediate vicinity of the site of penetration.

Sporulation and Oospore Formation on *Arabidopsis* Strain Weiningen

Seven days post-inoculation both asexual and sexual reproduction of *P. parasitica* were observed in infected leaves of *Arabidopsis* strain Weiningen. Conidiophore primordia developed from the apices of comparatively very broad hyphae in substomatal cavities. Figure 3A shows that conidiophores were similar in height to the leaf trichomes. Conidiophores showed a marked constriction in the region of the stoma (Figure 3B), and two conidiophores were often seen emerging from a single stoma. After growth out of the stomata (Figure 3C), conidiophores expanded, elongated, and quickly adopted a tree-like shape. Vesicles appeared at the end of each branch and expanded to form conidia. Mature conidia had a smooth to slightly verrucose surface (Figure 3B). Sexual reproduction started with the intertwining of hyphae, which then differentiated to form oogonia and antheridia. Antheridia were of the paragynous type and appeared firmly appressed to oogonia (Figure 3D). Mature oospores were present in great number by 8 days after inoculation (Figure 3E). Their formation apparently coincided with the onset of sporulation.

Symptom Expression

Before the onset of sporulation, infected leaves remained macroscopically free of disease symptoms. Sporulation of the fungus occurred 6 days after inoculation, after incubation of plants in the dark in a moist chamber for approximately 16 hr. Conidiophores grew singly from stems and leaf petioles. Thick tufts of conidiophores were observed on the abaxial and adaxial side of leaves, as seen in Figure 4. Heavily infected plants died within 1 to 3 days. No sporulation or macroscopically visible symptoms were induced on inoculated plants of *Arabidopsis* strain RLD.

DISCUSSION

Our microscopic and macroscopic observations of the development of *P. parasitica* on *A. thaliana* are in full agreement with recent reports dealing with downy mildew of crucifers. The preferential development of appressoria at the junction between anticlinal walls of adjoining epidermal cells has been reported for *P. parasitica* (Preece et al., 1967; Chou, 1970; Greenhalgh and Dickinson, 1975) and other members of the Peronosporacae (Tommerup, 1981). In addition, growth of the penetrating hypha of *P. parasitica* between anticlinal walls of epidermal cells and formation of the first haustorium therein have been reported previ-

Figure 2. (continued).

(A) Hypersensitive reaction. Two necrotic epidermal cells adjacent to the penetration hypha (ph). Because of collapse of the epidermal cells, the outline of underlying mesophyll cells is visible (arrows). Bar = 10 μm.

(B) Hypersensitive necrosis of a single epidermal cell after penetration of the hypha through the anticlinal wall. Letters a and c refer to appressorium and conidium, respectively. Bar = 10 μm.

(C) and **(D)** Penetration hypha (ph) growing between palisade cells at two different depths of focus. Haustoria are not formed. The cytoplasm in the two cells in contact with the hypha differs in appearance from that of the surrounding cells. Note the reduction in diameter of the hypha toward the tip. Bar = 10 μm.

(E) to **(H)** Development of the fungus at a single penetration site, documented by varying the depth of focus. Note that no haustoria are present.

(E) Conidium (c), germ tube (gt), and appressorium (a). Bar = 10 μm.

(F) The penetration hypha (ph) can be seen growing between the anticlinal epidermal cell walls. The cell contents adjacent to the penetration hypha appear granular (arrows). Bar = 10 μm.

(G) and **(H)** Growth of the penetration hypha (ph) in the mesophyll. Material has been deposited by cells at the point of contact with the fungus (arrows). Bar = 10 μm.

Figure 4. Sporulation of *P. parasitica* on Leaves of *Arabidopsis* Strain Weiningen (Viewed under a Stereo Microscope).

A lawn of conidiophores is present on the leaves (thick arrows). On petioles the conidiophores are formed singly (thin arrows).

ously on several hosts (Chou, 1970; Greenhalgh and Dickinson, 1975). Intercellular mycelial growth of *P. parasitica*, haustorium formation, and production of sexual and asexual organs in tissue of *Arabidopsis* all concur with previous studies on peronosporaceous fungi (Channon, 1981; Michelmore, 1981; Tommerup, 1981).

The only structures observed by light microscopy that indicated an attempt by the host to limit infection were encased haustoria. Encapsulation of haustoria in epidermal cells of the *Arabidopsis* Weiningen strain was a rare event. However, when encapsulation of a haustorium occurred in the epidermal cells, further development of the affected haustorium was suppressed. Successful infection of the host, however, was not stopped by this reaction. In contrast, in the mesophyll cells, encased haustoria were generally full sized. These observations suggest that formation of these encasements was not part of an effective defense reaction.

Completely ensheathed, apparently full-grown haustoria of *P. parasitica* were also occasionally observed by Chou (1970) on cabbage. Based on ultrastructural studies, Sargent (1981) reported that in infections of lettuce with the downy mildew fungus *Bremia lactucae* deposition of callose was a continuous process, resulting in complete encasement of old haustoria.

The absence of host cell necrosis, the rapid spread of the intercellular mycelium, a high frequency of haustorium formation, and the production of conidiophores and oospores all indicated a high degree of compatibility between host and parasite. Thus, the *A. thaliana* Weiningen strain proved to be highly susceptible to the downy mildew fungus *P. parasitica*. In contrast, plants of the *Arabidopsis* RLD strain appeared to be completely resistant to *P. parasitica*, with penetrated cells undergoing a typical hypersensitive response (Figures 2A and 2B). This opens the question of the potential of this host-pathogen interaction as a model system for genetic and molecular biological studies on resistance/susceptibility. In an ideal model system, both host and parasite are easy to culture and amenable to genetic analysis. In this respect, the virtues of *Arabidopsis* are clear (Meyerowitz, 1989; Somerville, 1989). *P. parasitica*, however, is a biotroph and cannot be grown on artificial media. Although various methods of culturing are known, they all require the use of living, susceptible tissue (Channon, 1981). The second disadvantage is the lack of detailed knowledge of the sexual system in *P. parasitica*. The existence of heterothallic and homothallic isolates has been demonstrated (DeBruyn, 1937), and McMeekin (1960) showed that separate antheridial and oogonial strains of *P. parasitica* exist. Infection of seedlings from soil-borne oospores has been reported (Channon, 1981), but attempts by McMeekin (1960) to induce germination of oospores were unsuccessful.

Nevertheless, the *P. parasitica*/*A. thaliana* interaction has several promising features. Being a typical biotroph, *P. parasitica* generally shows a high degree of host specificity. Specialization of parasitism in *P. parasitica* may be exhibited at the generic, specific, and lower taxonomic

Figure 3. Asexual and Sexual Reproductive Structures of *P. parasitica* in and on Tissues of *A. thaliana* Strain Weiningen.

Samples were taken 7 days after inoculation.

(A) Conidiophore (cp) emerging from the leaf surface; the conidia are partly discharged. Conidiophore and trichome (t) are similar in length. Bar = 45 μm.

(B) The base of a conidiophore (cp) and several discharged conidia lying on the leaf surface. Note the constriction of the conidiophore in the stomatal opening (arrow). Conidia have a smooth to slightly verrucose surface. Bar = 5 μm.

(C) A conidiophore initial (ci) growing out of a stoma and branching. Two conidiophore initials are apparently growing out of the neighboring stoma (arrows). Bar = 10 μm.

(D) An oogonium (o) with a paragynous antheridium (an) can be seen in the mesophyll. h and ih, haustorium and intercellular hypha, respectively. Bar = 25 μm.

(E) An oogonium (o) with an antheridium (an) attached, and mature oospores. The different structural layers of the mature oospores are clearly visible. h, haustorium; ih, intercellular hypha; osp, oospore; osw, oospore wall; ow, oogonial wall; pe, periplasm. Bar = 25 μm.

levels of the host (Channon, 1981). Little is known about the genetic control of resistance to *P. parasitica* in the Cruciferae, but Lucas et al. (1988) recently reported the identification of a gene for race-specific resistance to *P. parasitica* in *Brassica napus* (oilseed rape). In the present study, *Arabidopsis* strain Weiningen was highly susceptible to the isolate of *P. parasitica* used, whereas observations of inoculated plants of strain RLD indicated complete resistance. Inoculation of a number of *Arabidopsis* strains with a number of isolates of *P. parasitica* should reveal whether there is specialization of the pathogen into physiological races that can be differentiated by the reactions on different strains of *Arabidopsis*, and/or whether there is simply a gradient of virulence with respect to different host strains, i.e., interactions ranging continuously from immunity to high susceptibility. A large number of *Arabidopsis* ecotypes and mutants are available from a central international seed collection (Kerchheim and Kranz, 1985). It is most likely that *P. parasitica* infections on *Arabidopsis* occur in other countries and locations. It must be strongly suspected that pathotypic variation will exist in such geographically widely separated populations.

METHODS

Plant and Fungal Material

Arabidopsis plants with downy mildew infections were observed in May 1989 in an oilseed rape field near Zürich. Five to 10 plants were dug from the soil, placed in pots, and kept in the glasshouse, where they were incubated overnight in a moist chamber to promote sporulation. Leaves, stems, and pods bearing conidiophores of *Peronospora parasitica* were removed from the plants and stored in glass vials at −20°C. Plants were allowed to set seed which was further increased. This plant material is referred to as *Arabidopsis* strain Weiningen in this report. *Arabidopsis* strain RLD was kindly supplied by Werner Bernhard (this institute).

Cultivation of Plants

Arabidopsis was grown in 12-cm-diameter plastic pots in potting compost covered with a layer of fine vermiculite. After watering the soil, seeds were sown densely enough onto the vermiculite to give a lawn of plants. Pots were then covered with Saran Wrap and kept up to 4 weeks at 4°C until required. Plants were grown in a glasshouse at 23 ± 3°C. Additional lighting (16 hr) was supplied by a high-pressure sodium lamp. In this environment, germination of seeds occurred within 3 to 4 days, after which the Saran Wrap was removed from the pots.

Inoculation of Plants

Plants were inoculated 2 to 3 weeks after germination when four to five true leaves were present. For the initial inoculation, tissue that had been stored at −20°C for 5 months was thawed at room temperature, and a few milliliters of water were added. After vortexing, the resulting suspension was passed through two layers of cheesecloth and sprayed with a chromatographic sprayer onto the plants. Plants were incubated overnight at 20°C in a moist chamber and returned to the glasshouse for disease development. Five days after inoculation, plants were again incubated overnight in the moist chamber to promote sporulation of the fungus. After the fungal culture was established, an easier method of inoculation was employed. After moist incubation, plants bearing conidiophores were simply rubbed against uninoculated plants, thus depositing spores onto the latter. The freshly inoculated plants were again incubated in the moist chamber. This method was convenient for routine maintenance of pathogen stocks and resulted in high infection densities that were particularly useful for the histological studies of the infection process.

Light Microscopy

Infection and development of the fungus were studied in whole-leaf mounts stained with lactophenol-trypan blue (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, 10 mg of trypan blue, dissolved in 10 mL of distilled water) (based on Keogh et al., 1980). Whole leaves were boiled for approximately 1 min in the stain solution and then decolorized in chloral hydrate (2.5 g of chloral hydrate dissolved in 1 mL of distilled water) for at least 30 min. They were mounted in chloral hydrate and viewed under a compound microscope equipped with interference or phase-contrast optics.

Scanning Electron Microscopy

Infected leaves were fixed in the vapor of a 4% (w/v) aqueous solution of osmium tetroxide for 3 hr at room temperature, dehydrated in a graded series of acetone, and critical point dried. After mounting on specimen stubs with conductive silver print paint and sputter-coating with an 80%:20% alloy of gold and palladium, the samples were examined in a Cambridge S-4 Stereoscan electron microscope.

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