Arabidopsis Is Susceptible to Infection by a Downy Mildew Fungus

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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 pathogen 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 Stenophagrum 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.

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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 were related to observations by Chou (1970), who concluded from ultrastructural studies that epidermal cells of cabbage were resistant 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 from the host's cell wall.

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 \( \mu m \).

(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 \( \mu m \).

(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 \( \mu m \).

(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 \( \mu m \).

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

(F) Encasement (arrows) in epidermal cells surrounding haustorial initials. aw, anticlinal walls. Bar = 10 \( \mu m \).

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

(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 \( \mu m \).

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

(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 \( \mu m \).
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 adhered 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 Peronosporaceae (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 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).

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 chlorhydrate 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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