An Elicitor from *Botrytis cinerea* Induces the Hypersensitive Response in *Arabidopsis thaliana* and Other Plants and Promotes the Gray Mold Disease

Eri M. Govrin, Shimon Rachmilevitch, Budhi Sagar Tiwari, Mazal Solomon, and Alex Levine

Department of Plant Sciences, The Hebrew University of Jerusalem, Givat-Ram Jerusalem 91904, Israel.
Accepted for publication 3 October 2005.

ABSTRACT

Govrin, E. M., Rachmilevitch, S., Tiwari, B. S., Solomon, M., and Levine, A. 2006. An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. *Phytopathology* 96:299-307.

Botrytis cinerea is a necrotrophic fungus that infects over 200 plant species. Previous studies showed that host cells collapse in advance of the hyphae, suggesting secretion of toxins or elicitors. We have partially characterized elicitor activity from intercellular fluid extracted from *Arabidopsis thaliana* leaves infected with *B. cinerea*. Treatment of intact leaves or cell cultures with either intercellular fluid from infected leaves or medium from inoculated *A. thaliana* cell culture induced generation of reactive oxygen species, resulting in reduced photosynthesis, electrolyte leakage, and necrotic lesions that resembled the hypersensitive response (HR). The necrosis was inhibited by diphenyleneiodonium, a specific in-

hibitor of NADPH oxidase, and by chelating free iron, suggesting the involvement of hydroxyl radicals. The necrosis was also suppressed in *dnd1* mutants that are compromised in HR. In contrast, increased cell death was observed in *acd2* mutants, indicating the involvement of the host defense signaling pathways. Treatment with the intercellular fluid from infected leaves also induced transcription of pathogenesis-related (PR) genes, such as *PR-1*, *PR-5*, *HSR203J*, and of senescence-associated gene *SAG-13*. Moreover, rapid transcription of the ethylene-dependent *AtEBP* gene was detected, indicating induction of ethylene production. The intercellular fluid from infected *A. thaliana* induced cell death in other plants, in line with the lack of *B. cinerea* specificity. In summary, the intercellular fluid mimicked a range of molecular and physiological host responses that are observed during infection with a live fungus. Moreover, it accelerated the *B. cinerea* infection, suggesting that the elicitor may act as a pathogenicity factor in the progression of gray mold disease.

Botrytis cinerea is a nonspecific necrotrophic pathogen that attacks more than 200 plant species (39). *B. cinerea* infection is first noticeable by the appearance of small necrotic lesions at the site of inoculation. Following the primary infection, the lesions increase in size as the fungus becomes established (3). The killing of plant cells is essential for *B. cinerea* growth (13,52).

During evolution, plants have developed efficient strategies to respond to attempted infections. The perception of pathogen attack that takes place by detection of microbial products, called elicitors or pathogen-associated molecular patterns, is critical for mounting a successful defense (58). Many fungi attack plants by producing general or host-specific toxins (14). Traditionally, compounds that caused rapid host cell death in the absence of detected plant defense responses were classified as toxins. In contrast, the elicitors, although also capable of triggering host cell death, are considered inducers of defense responses. Recent studies of a well-characterized toxin from *Cochliobolus victoriae*, victorin, which causes host cell death, revealed that it induced phytoalexin biosynthesis and other defense responses that are usually associated with elicitors (28). Moreover, inhibitors of cell death signal transduction suppressed cell death caused by victorin. Similar induction of cell death concomitant with activation of defense reactions was recently described in *Arabidopsis thaliana* in response to fumonisin B1 mycotoxin (45) and in tomato treated with toxins from *Alternaria alternata* (53). Importantly, toxin-induced cell death could be suppressed by expression of antiapoptotic

genes, such as *Bcl-2* and *Bcl-xl*, indicating suicide-like cell death (11,55).

Toxin production by *B. cinerea* has been suspected for many years, although its presence was never clearly demonstrated (20, 39). Gentile showed in 1951 that a thermostable, nonvolatile factor from *Exochorda racemosa* plants infected with *B. cinerea* could induce lesions similar to the live fungus infection (15). A phytotoxic polysaccharide factor was isolated from *B. cinerea* broth and shown to produce disease symptoms (50). More recently, a number of toxic sesquiterpene molecules, such as botrydial, have been purified from shaking cultures of *B. cinerea* (6,40). However, the level of botrydial in leaves infected by different isolates did not correspond to the disease susceptibility, leading the authors to conclude that it is not a primary determinant of pathogenicity (8). This conclusion was recently corroborated by the deletion of the *B. cinerea* gene that encodes a P450 monooxygenase in the botrydial pathway, which showed only minor effect on virulence (43).

As early as 1888, Ward noticed that host cells collapsed in advance of *B. cinerea* hyphae (54). A similar conclusion was reached several years later by Smith (44). We have confirmed these observations in *A. thaliana* leaves, and showed that cells at a considerable distance from the growing hyphae were accumulating H₂O₂ before cell death (16). Generation of reactive oxygen species (ROS) has been documented in numerous plant-pathogen interactions (22) and shown to play a key role in the hypersensitive cell death during pathogenesis (10,24,32). Moreover, *B. cinerea* disease was suppressed by pretreatment with diphenyleneiodonium (DPI), an inhibitor of the plasma-membrane NADPH oxidase, indicating a critical role for host-generated ROS (16). The above observations suggested that *B. cinerea* may be secreting a factor that elicits an oxidative burst which triggers an active (programmed) cell death in the host.

Corresponding author: A. Levine; E-mail address: AlexLevine@huji.ac.il

Here we describe the effects of *B. cinerea* elicitor from infected plants. We show that the cell-free intercellular fluid from *A. thaliana* leaves infected with *B. cinerea* mimicked the full range of plant responses to the fungal infection, including the oxidative burst, induction of defense gene transcription, and hypersensitive cell death.

MATERIALS AND METHODS

Materials. 2,7-Dihydrodichlorofluorescein was obtained from Molecular Probes Inc. (Eugene, OR), DAF-2 from Alexis Biochemicals (San Diego, CA), and 4',6-diamidino-2-phenyl-indole (DAPI) from Calbiochem (San Diego, CA). Zn^{2+} desferrioxamine was a gift from M. Chevion (Hadassah, Jerusalem, Israel), and DPI and other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Plant and fungal cultures. *A. thaliana* plants var. Col-O were grown for 4 to 5 weeks in growth chambers at 23°C with 16 h light. Cell cultures were provided by C. J. Leaver (University of Oxford, UK). Cultures were kept at 25°C under constant light in Murashige and Skoog's medium supplemented with myo-inositol (100 mg/liter), thiamine (0.4 mg/liter), naphthalene acetic acid (0.5 mg/liter), kinetin (0.05 mg/liter), and 3% sucrose. *A. thaliana* cultures were diluted fivefold every week and used 2 days after the transfer. *B. cinerea* (strain INRA) was provided by A. Mayer (The Hebrew University) and grown in constant light at 20°C on potato dextrose agar as previously described (17).

Preparation of intercellular fluid from infected leaves and cell-free medium from suspension-cultured cell. Spores of *B. cinerea* were collected and prepared as described in Govrin and Levine (17). The spores were diluted to the concentration of 20 spores per μl , and the inoculation was done by placing a drop of 5 μl in the center of the infiltrated area.

To prepare the intercellular fluid, *A. thaliana* plants were sprayed with *B. cinerea* spores at a concentration of 10^5 spores per ml with a compressed air sprayer. Plants were kept for 3 days at 20°C in plastic boxes covered with nylon under constant light. Twenty leaves were put into 12.5 ml of water in a 50-ml syringe and subjected to five alternating vacuum and air pressure regimens. The material was filtered through 0.2 μm and kept at -20°C. To estimate the endogenous concentration of the elicitor, the apoplastic water volume was determined according to Husted and Schjoerring (19), and was calculated to correspond to about 11% of the fresh leaf weight. Unless otherwise indicated, in all experiments the intercellular fluid was diluted 300-fold. For control, the apoplastic fluid was extracted from uninfected leaves in an identical manner. No necrotic symptoms were observed at any concentration of the control fluid.

The cell-free medium from *A. thaliana* suspension culture infected with *B. cinerea* was prepared by inoculation of 10^4 spores of *B. cinerea* into a 250-ml flask filled with 40 ml of a 5-day-old *A. thaliana* culture. The medium was collected after 8 days, and the cultures were washed with 40 ml of water. The culture fluid and the water-extracted material were filtered separately through 0.2- μm filters (Sartorius, Germany), and activity was tested on *A. thaliana* cells. Since the activity of the water-extracted fraction was routinely 85 to 90% of the culture medium, both fractions were pooled together. Infiltration into leaves was done with a 1-ml syringe as described in Govrin and Levine (17).

ROS measurements. ROS were assayed as described in Lu and Higgins (26). A minor modification was made in the method of sample preparation: leaves were removed 6 h after treatment, washed briefly in distilled water, and placed into glass tubes with 0.1 M phosphate buffer, pH 8, and 2,7-dihydrodichlorofluorescein. Aliquots were withdrawn and analyzed in a fluorometer (BioTek FL600; Bio-Tek Instruments, Winooski, VT) at 485 nm excitation/525 nm emission wavelengths.

Photosynthesis measurements. Gas exchange of fully developed, attached leaves of *A. thaliana* was determined with a Model LCA-2 system (Analytical Development Company, Hoddesdon, England). For these measurements, different mixtures of oxygen, nitrogen, and carbon dioxide were obtained with two cascaded gas-mixing pumps (Model 2CO-18-2F; Wosthoff Co., Germany). Leaf gas exchange parameters were measured or derived by standard procedures (51). CO_2 assimilation/internal leaf CO_2 partial pressure (A/C_i) values were determined from gas exchange measurements made under 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux.

Northern analysis. RNA preparation, blotting, and hybridization were as described in Govrin and Levine (17). The tobacco *HSR203J* and the *AtEBP* probes were generous gifts from D. Roby (INRA, Castanet-Tolosan, France) and J. Ecker (Salk Institute, La Jolla, CA), respectively. *PR-1* and *PR-5* were from K. Shirazu (Salk Institute), *PDF-1.2* from J. Dangl (University of North Carolina), and *GST-1* from J. Greenberg (Boston Gen. Hosp., Boston, MA).

Fluorescent microscopy. For nuclear staining, leaves were incubated for 15 min in 5 μM DAPI and viewed with an Olympus IX70 fluorescence microscope at 365HT25/450DF65 nm excitation/emission wavelengths. Chlorophyll autofluorescence was viewed at 535DF/OG590 nm wavelengths as described in Mazel and Levine (29). For ROS observation, leaves were incubated in 0.2 ml of 10 μM DCFH-DA for 15 min in the dark in phosphate buffer, pH 7.5, and viewed at 485DF22/OG530 nm excitation/emission wavelengths.

Conductivity assay. A total of six leaf disks, 1 cm in diameter, were excised from the third and fourth rosette leaves from six different plants, rinsed in water for 10 min, and floated on the abaxial side on 3 ml of double-distilled water for 3 h at room temperature. Conductivity was measured with a Consort K511 Conductometer (Scientific Instruments, Tumhout, Belgium).

RESULTS

Induction of necrosis by intercellular fluid from leaves infected with *B. cinerea*. As a first step to examine possible production of elicitor or toxin by *B. cinerea*, we collected intercellular fluid from infected *A. thaliana* leaves and infiltrated it into leaves of naive plants. The volume of intercellular fluid was estimated by the vacuum infiltration technique, which extracts the apoplastic solution with very little cytoplasmic contamination (19). Dark, water-soaked lesions resembling *B. cinerea* infection began to develop within 8 to 24 h after infiltration of the intercellular fluid. A rapid necrosis occurred after infiltration of a twofold dilution of the intercellular fluid that spread beyond the initial infiltration area (Fig. 1A, leaf 1), and a typical necrosis, that was confined to the infiltrated area, developed within 24 to 36 h, even after 200-fold dilution of the intercellular apoplastic concentration (Fig. 1A, leaves 2 and 3). The symptoms produced by a 300-fold dilution of the intercellular fluid developed more slowly and were weaker, and a 5,000-fold dilution did not produce any visual symptoms even after 3 days (Fig. 1A, leaf 4). No symptoms were observed after infiltration of intercellular (control) fluid, which was extracted from uninfected plants (data not shown), at any concentration.

Induction of necrosis occurred at concentrations considerably below those present in infected tissue. The intercellular fluid moved with the transpiration stream when detached leaves were placed in water with the intercellular fluid (Fig. 1B). Infiltration of the medium from inoculated cell culture into *A. thaliana* leaves produced lesions as effectively as intercellular fluid (data not shown).

The onset of cell death was assayed by measuring ion leakage, which indicates cellular collapse (46). Cells started to die 8 h after infiltration of the intercellular fluid (Fig. 1C), which preceded the

appearance of necrotic lesions by several hours. Staining of the treated leaves with DAPI revealed fluorescent nuclei in the treated area, indicating chromatin condensation which is often associated with programmed cell death (Fig. 2A) (23,36). The DAPI fluorescence observed in the infiltrated area coincided with reduced chlorophyll autofluorescence and reduced photosynthetic capacity in the treated area, indicating early onset of chlorophyll damage (Fig. 2B and C). Infiltration of intercellular fluid caused a 20% decrease in photosynthesis as measured by reduced photosynthetic carbon dioxide (CO₂) uptake after 1 h following the infiltration and reached 50% inhibition by 4 h. The change in CO₂ concentrations per unit time is correlated with net photosynthesis (assimilation). The earliest visible necrotic symptoms appeared after more than 12 h.

To test whether the cell death caused by the intercellular fluid from infected leaves utilized the programmed cell death signaling pathways of the host, the intercellular fluid was infiltrated into two *A. thaliana* mutants that exhibit opposing phenotypes with regard to induction of cell death during pathogenesis. We have shown previously that the *dnd1* mutants, which do not exhibit hypersensitive cell death to avirulent strains of *Pseudomonas syringae* (56), were also less susceptible to infection with *B. cinerea* (16). Infiltration of the intercellular fluid from leaves infected with *B. cinerea* into the *dnd1* leaves was symptomless (Fig. 3A), consistent with the results of live fungus infection (16). On the other hand, infiltration of the intercellular fluid into *acd2* (accelerated cell death mutant) plants induced a strong and rapid necrosis com-

Fig. 1. Induction of cell death (necrosis) in *Arabidopsis thaliana* by intercellular fluid from leaves infected with *Botrytis cinerea*. **A**, Five-week-old *A. thaliana* plants were infiltrated with different dilutions of intercellular fluid from leaves infected with *B. cinerea* and leaves were photographed 32 h (leaves 2 to 4) or 96 h (leaf 1) after infiltration. Note that in leaf 1 the high concentration of the intercellular fluid caused spreading of the necrotic area with time. No spreading of necrosis was seen with the more diluted concentration, and no necrosis was seen at any concentration of the intercellular fluid from uninfected leaves. Infiltrations with the following dilutions are shown: leaf 1, 1:2; 2, 1:100; 3, 1:300; and 4, 1:5,000. The infiltrated area in all leaves (including leaf 1) was the same (≈ 5 mm). **B**, Four-week-old leaves were detached and their petioles placed into water with or without intercellular fluid (300-fold dilution). Chlorosis followed by necrosis developed in leaves incubated in the intercellular fluid (IF) within 2 days. No chlorosis was observed in the leaves placed into intercellular fluid from mock-(control) inoculated leaves (CF) even after 4 days. **C**, Measurements of ion leakage following infiltration of intercellular fluid from infected leaves. Leaves were infiltrated with a 300-fold dilution of the intercellular fluid. At the indicated time points, leaves were detached and conductivity was measured. No symptoms were seen during the first 15 h after infiltration. Cell death was assayed by measuring ion leakage from leaf disks. The bars indicate standard deviation ($n = 6$). ◆, intercellular fluid from infected leaves; and ■, intercellular fluid from healthy leaves (control).

pared with wild-type plants, where the symptoms started to appear at 48 h.

Since *B. cinerea* is a nonspecific pathogen, it was interesting to test whether the intercellular fluid isolated from infected *A. thaliana*

plants induced necrosis in other species. Infiltration of the intercellular fluid from infected leaves produced necrotic lesions in bean and tobacco leaves (Fig. 3B). Similar results were obtained by infiltration of the cell-free medium from infected cell

Fig. 2. Characterization of cell death caused by intercellular fluid in wild-type *Arabidopsis thaliana* plants. **A**, Condensation of chromatin in cells treated with intercellular fluid from infected leaves (left) or from control leaves (right). Intact leaves were infiltrated with intercellular fluid (300-fold dilution) and stained with 4',6-diamidino-2-phenyl-indole (DAPI) 12 h later. The ring delineates the infiltrated area. The picture shows fluorescence of DAPI only in the infiltrated area on the left (the center of infiltration is outside the picture frame), while no signal is seen in the healthy part of the leaf (right) or in the control treated leaves (right). **B**, Chlorophyll autofluorescence in the leaf treated as in **A**, showing damage to the chlorophyll in the infiltrated area. The right image shows a control treated leaf. Scale bar is 100 μm . **C**, The effect of intercellular fluid on photosynthesis of *A. thaliana*. Leaves were infiltrated with intercellular fluid (IF) from infected leaves and the net CO_2 gas exchange was measured after 1 and 4 h. Measurements were taken under $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux. Black bars, leaves treated with the intercellular fluid from infected leaves; white bars, control fluid.

Fig. 3. Induction of cell death in mutant *Arabidopsis thaliana* and other plants. **A**, Treatment of wild type, *acd2*, and *dnd1* mutant *A. thaliana* plants with intercellular fluid. Leaves were photographed 33 h after treatment. In the wild-type plants the symptoms appeared within 48 h, while in the *acd2* mutants they were visible already after 24 h, and in *dnd1* mutants no symptoms were seen even 4 days after infiltration. **B**, Bean and tobacco plants were infiltrated with intercellular fluid from leaves infected with *Botrytis cinerea* or from leaves treated with water (control). No symptoms were seen in either plant when the leaves were infiltrated with water (data not shown). Plants were photographed 44 h after infiltration.

culture from *A. thaliana*, but neither the intercellular fluid from the uninfected leaves nor cell cultures showed any visible symptoms (data not shown).

Characterization of the *B. cinerea* elicitor activity. Preliminary characterization of the necrotizing activity of the intercellular fluid from leaves infected with *B. cinerea* showed that it was caused by a small molecule that diffused through a 1-kDa cutoff dialysis membrane. Fractionation of the intercellular fluid from the infected leaves with an equal volume of ethyl acetate recovered the activity in the aqueous phase. Macromolecules were also removed by sequential precipitation with 70% and then with 95% ethanol and centrifugation. The pellet and the supernatant were lyophilized and resuspended in water and infiltrated into *A. thaliana* leaves. Almost 100% of the activity was retained in the supernatant. The active fraction was heat stable (10 min at 95°C) and resistant to treatments with proteinase K and RNase A (data not shown). More detailed analysis will be necessary to identify the molecular structure of the active compound.

Induction of ROS by cell-free intercellular fluid from leaves infected with *B. cinerea*. One of the earliest plant responses to pathogens is the generation of ROS (22). The oxidative burst was detected within several hours of *A. thaliana* infection by *B. cinerea* (16,52). It was therefore interesting to determine whether ROS production occurred in response to treatment with the cell-free intercellular fluid from leaves infected with *B. cinerea*. Increased ROS accumulation was observed in the area infiltrated with intercellular fluid from infected medium but not with the control medium (Fig. 4A, inset). Quantitative measurements confirmed the microscopy data (Fig. 4A). Infiltration of catalase abolished the fluorescence, verifying that the major ROS in the treated leaves was H₂O₂, in line with previous results from fungal infection (16).

To test the role of the ROS production in the necrosis development, the *A. thaliana* leaves were treated with DPI, an inhibitor of the plasma membrane NADPH oxidase (31,33). The addition of DPI to the intercellular fluid from infected leaves effectively suppressed the formation of lesions, implicating the NADPH oxidase in the production of ROS and the formation of lesions (Fig. 4B). Since iron is a potent facilitator of hydroxyl radical production via the Fenton reaction (especially in the presence of H₂O₂), and also by virtue of hypervalent iron compounds, we tested whether

free iron played a role in the development of necrosis. The free iron was chelated with a complexed desferrioxamine (42,57), which was infiltrated together with the intercellular fluid. Addition of the iron chelator strongly suppressed the formation of lesions, suggesting a possible involvement of the hydroxyl radicals in necrosis (Fig. 4B).

Induction of plant defense genes by intercellular fluid. The results presented above indicate that plants perceived the presence of a soluble *B. cinerea* elicitor that activates the hypersensitive cell death pathway. To see whether the perception of intercellular fluid also triggered the signal transduction pathways that lead to the induction of defense gene transcription, we analyzed gene expression using probes from representative defense pathways that are commonly induced during pathogenesis (48). As shown in Figure 5A, the intercellular fluid from infected leaves induced defense genes from several pathways. These genes were shown earlier to be induced by the inoculation of *B. cinerea* spores, suggesting that plant cells activated the same signaling pathways in response to the intercellular fluid from infected leaves as when challenged with the live fungus (16). It is notable, that in tobacco the intercellular fluid induced the expression of the *HSR203* gene, which is considered a hypersensitive response (HR)-specific gene (37,38). Moreover, the *HSR203* gene was induced very early, as described for the HR-specific expression of this gene in tobacco plants challenged with several incompatible pathogens. The intercellular fluid from infected leaves also induced expression of the *AtEBP* gene, indicating a rapid increase in ethylene biosynthesis (Fig. 5B).

The effect of intercellular fluid on gray mold disease caused by *B. cinerea*. To test whether the elicitor participated in the development of *B. cinerea* disease, we treated the *A. thaliana* leaves with the cell-free intercellular fluid from infected leaves and then inoculated them with a relatively low number of *B. cinerea* spores (20 spores per ml, which is fivefold less than the concentration used for rapid infection [16,17]). Substantially larger lesions were detected in plants that were infected with *B. cinerea* after treatment with the intercellular fluid from infected leaves, as opposed to infection following infiltration of intercellular fluid from mock-infected leaves (Fig. 6A). The increased lesions size was correlated to increased fungal growth, as tested by the expression of a constitutive *B. cinerea*-specific actin gene (Fig. 6B).

Fig. 4. Production of reactive oxygen species (ROS) in *Arabidopsis thaliana* leaves treated with intercellular fluid. **A**, *A. thaliana* leaves were infiltrated with deionized distilled water (DDW) or with intercellular fluid from control (CF) or infected (IF) leaves. Where indicated, catalase (cat) was included to verify the presence of H₂O₂, at a concentration of 1,000 units/ml. ROS were assayed 6 h after treatment with 2,7-dihydrodichlorofluorescein as described in Lu and Higgins (26). The experiment was repeated four times with similar results. Each data point is the average of four replicates; the error bars represent standard deviations. The inset shows development of ROS-dependent fluorescence (appearing as bright color after conversion to grayscale) as viewed by epifluorescence microscopy. Arrow indicates the center of infiltration. **B**, Suppression of necrosis by iron chelation or by inhibition of the NADPH oxidase. Intact *A. thaliana* leaves were infiltrated with the cell-free intercellular fluid (IF) from leaves infected with *Botrytis cinerea* together with 100 μ M Zn²⁺ desferrioxamine (MCJ), an iron chelator (42), or with 3 μ M DPI, an inhibitor of membrane NADPH oxidase (33). Leaves were detached and photographed 32 h after treatment.

DISCUSSION

In this paper, we have investigated the cause of the oxidative burst and cell death in infected host tissue that occurs at a considerable distance in advance of growing *B. cinerea* hyphae (9,16). The production of toxin by *B. cinerea* has been proposed some 50 years ago (15), but to date, no convincing evidence for the existence of a toxin has been shown (39). Several extracellular polygalacturonases have been isolated and shown to act as virulence factors, but mutants of *B. cinerea* in these genes were still pathogenic and exhibited identical primary infections (39,47). Phytotoxic compounds of low molecular weight, such as botrydial, were previously isolated from shaking or standing liquid cultures of *B. cinerea* (12,40). However, botrydial and other toxins from the *B. cinerea* cultures were never identified in plant tissues

infected with isolates of *B. cinerea*. In this work, we partially characterized a potent elicitor of cell death isolated from *Arabidopsis* leaves inoculated with *B. cinerea*. The elicitor described here exhibited a strongly hydrophilic nature (partition into the aqueous phase in the ethyl acetate extraction) that is in contrast to botrydial, which belongs to the bicyclic sesquiterpenoid class that is highly lipophilic. The described elicitor also differs from the polysaccharide factor isolated by Vannell et al. (50) since it is soluble in ethanol.

The source of the elicitor has not been addressed in this study. Theoretically, the elicitor/toxin present in the intercellular fluid from the infected leaves could be produced by *B. cinerea* or by *A. thaliana* cells that react against *B. cinerea*. Nonetheless, the primary source of the physiological and molecular effects described here must be triggered by the fungus.

Fig. 5. Analysis of defense gene expression in *Arabidopsis thaliana* leaves treated with intercellular fluid. **A**, Intact leaves of *A. thaliana* or tobacco plants were infiltrated with the cell-free intercellular fluid from *A. thaliana* plants that were infected with *Botrytis cinerea* (IF) or mock-treated (CF). Intercellular fluid from untreated leaves was used as an additional control (cont). Total RNA was extracted after 24 h (IF-24) or after 40 h (IF-48). The gene expression that was induced by the intercellular fluid was compared with the expression patterns in plants infected with *B. cinerea* (*B. cin*) after 48 h. Blots were probed with indicated genes as described in Govrin and Levine (17). The *HSR203* expression was probed in tobacco plants 6 h (IF-6) and 14 h (IF-14) after treatment, as this is an early hypersensitive response-specific gene of tobacco. **B**, Activation of the ethylene signaling pathway tested by the expression of *A. thaliana* ethylene binding protein *AtEBP*. RNA was extracted at the indicated times after infiltration of intercellular fluid from infected *A. thaliana* and analyzed by northern blotting with *AtEBP* probe.

Fig. 6. The effect of intercellular fluid from infected leaves on *Botrytis cinerea* disease. **A**, Four-week-old *Arabidopsis thaliana* plants were infiltrated with the intercellular fluid from infected leaves (IF) or from control leaves (CF). After 30 min, the leaves were inoculated with 100 spores of *B. cinerea* (per inoculation site, two sites in each leaf). The picture was taken 1 day after treatment. **B**, Northern analysis of *B. cinerea* growth in plants treated as in A. RNA was extracted 3 days after treatment and probed with a specific fragment of the constitutively expressed actin gene from *B. cinerea*, as previously described (16).

Dependence of the *B. cinerea* elicitor activity on the host background. *B. cinerea* is a nonspecific pathogenic fungus that infects many unrelated plant species, although large variations in pathogenicity exist, especially in the infection of healthy, unwounded plants, which are naturally less susceptible to the opportunistic fungi (27). Infiltration of the intercellular fluid from infected *A. thaliana* tissues on healthy *Arabidopsis*, tobacco, and bean plants caused necrosis on all plant species tested within 36 to 48 h, consistent with the nonspecific mode of attack of the fungus (Fig. 3B). In planta, the concentration that is required to induce necrosis is probably adjusted by the hyphal growth during the disease progression, in order to keep the toxic levels only a few cell layers ahead of the hyphal tip. Therefore, although the elicitor could move within the leaf, the lesion development was restricted (Fig. 1). Uncontrolled production and long-distance diffusion of the toxic factor would be counterproductive, since the dead tissue would desiccate prior to the fungus reaching the area to benefit from the nutrients.

B. cinerea is a heterokaryotic organism and consequently its genotypes are variable and susceptible to significant change. Indeed, individual isolates were shown to exhibit variable aggressiveness even on different cultivars of the same species (9,52). Importantly, induction of necrosis by the intercellular fluid from infected *A. thaliana* plants depended on the plant's genetic background and was correlated with HR induction in these plants. Thus, the *dnd1* mutants, which fail to mount HR to pathogens (56), produced only minor lesions, while *acd2* plants, which exhibit accelerated cell death when challenged with different pathogens (18), rapidly developed a strong necrosis reaction (Fig. 3A). These results are in line with increased cell death of the *acd2* mutants to the fumonisin B1 toxin (2). The results of the intercellular fluid treatments are also in agreement with the reported infection of these mutants with *B. cinerea* spores (16). Taken together, these results suggest that the *B. cinerea* elicitor activates the same cell death pathway as the live fungus and the bacterial *P. syringae* pathogens that were used to characterize those mutants.

The effect of *B. cinerea* elicitor on ROS generation. Accumulating evidence links the oxidative burst with plant cell death, including *B. cinerea*-induced hypersensitive cell death (16,22). The proposed mechanism that produces ROS during the HR includes the plasma membrane NADPH oxidase and a cell wall peroxidase (5). We have previously shown that *B. cinerea* infection induced a massive H₂O₂ accumulation, which extended to a considerable distance from the growing hyphae (16). The inhibition of ROS accumulation by infiltration of catalase indicates that H₂O₂ was the main ROS that was induced by the intercellular fluid (Fig. 4A). The accumulation of H₂O₂ relatively far from the growing hyphae is contrary to *P. syringae* infection, which induces H₂O₂ only in the cells in contact with the pathogen (4). This may be the result of the diffusion of the *B. cinerea* elicitor versus the interaction with the surface bacterial elicitors, such as flagellin or lipopolysaccharide of the outer membrane of gram-negative bacteria (58). The mechanism of the H₂O₂-dependent pathogenicity by necrotrophs has been suggested to involve the formation of hydroxyl radicals (7,21). The suppression of elicitor toxicity by the chelation of the free iron (Fig. 4B) is in agreement with the proposed mechanism of the *B. cinerea*-induced necrosis via the Fenton reaction.

The effect of *B. cinerea* elicitor on photosynthesis. The infiltration of intercellular fluid from infected leaves produced rapid physiological effects at the whole plant level before appearance of necrosis, as evidenced by the inhibition of photosynthesis already 1 h after treatment (Fig. 2C). Light, however, was not necessary for the observed cell death since both plants and cultured cells responded similarly in the dark to the intercellular fluid from leaves (or medium from cell cultures) infected with *B. cinerea*, albeit somewhat slower (data not shown).

Light is not essential for cell death induced by victorin, although the proteolytic degradation of Rubisco during the infection of *C. victoriae* indicated indirect involvement of chloroplasts (34). The biosynthesis of callose and the induction of ROS production lead Navarre and Wolpert, the authors of that study, to conclude that victorin possessed elicitor activity (34). Responses that are typical of elicitor treatments were also described in *A. thaliana* plants infiltrated with fumonisin (45).

The effect of *B. cinerea* elicitor on defense gene expression. The responses of plants to pathogen attack invariably involve changes in gene expression. One of the major groups of genes that are induced during pathogenesis belong to the class of pathogenesis-related (PR) proteins (25,35). Analysis of gene expression after treatment with the cell-free intercellular fluid from infected leaves revealed induction of several defense pathways. These included induction of the *PR-1* and *PR-5* genes, which are activated via the salicylic acid-dependent pathway (48), and the general stress inducible *GST-1* gene (Fig. 5A) (41). Especially important is the induction of the *HSR203J* gene in tobacco, which is considered an HR-specific marker in plants (37,38). Since the homologous gene has not been identified yet in *A. thaliana*, we assayed this gene in tobacco (Fig. 5A).

Interestingly, intercellular fluid induced the expression of a senescence-associated *SAG-13* gene (30) (Fig. 5A). Although there is evidence of some cross-talk between the cell death pathways during HR and senescence, these processes are regulated by distinct pathways, and the senescence-like process seems to be triggered at the periphery of the HR necrotic lesion (37). This suggests that the dying cells might release a signal able to induce senescence in the neighboring cells, which would help the *B. cinerea* infection. Furthermore, *B. cinerea* infection has been associated with ethylene accumulation, which also promotes plant senescence (49). Analysis of the expression of an ethylene inducible *AtEBP* gene (1) showed that it was induced as early as 1 h after infiltration of the intercellular fluid from infected leaves (Fig. 5B). The rapid induction of ethylene production suggests that the *B. cinerea* elicitor may promote the host senescence, which is advantageous to the fungus (13,39).

The effect of the *B. cinerea* elicitor on the development of gray mold disease. To assess the role of elicitor production on the pathogenicity of *B. cinerea*, the intercellular fluid infiltration was followed by inoculation of a small number of *B. cinerea* spores. Leaves that were pretreated with intercellular fluid from infected leaves, but not with the control fluid, exhibited accelerated appearance of necrosis, which is characteristic of gray mold disease (Fig. 6A). The visual disease symptoms were confirmed by analyzing the accumulation of a constitutively expressed *B. cinerea* actin gene (3) (Fig. 6B). The cell death process induced by the elicitor present in the intercellular fluid resembled HR in a number of aspects, such as early induction of the oxidative burst (Fig. 4B), chromatin condensation (36), and induction of the HR marker *HSR203J* gene (37,38).

In summary, our results show that *B. cinerea* produces an elicitor that triggers a range of host responses that are observed during infection with the live pathogen, including the hypersensitive cell death and defense gene expression. We suggest that the movement of the elicitor ahead of the growing hyphae facilitates the fungal ingress by promoting senescence and by initiating the cell death process, which is the hallmark of the gray mold disease. Purification of the elicitor and identification of its structure will enable the elucidation of its molecular mode of action.

ACKNOWLEDGMENTS

We thank A. Bent and J. Greenberg for providing the *dnd1* and the *acd2* mutants, respectively, and D. Roby for providing the probe of *HSR203J* gene. This work was supported by a grant from the EU FAIR5 project PL97-3351 and by Israel Ministry of Industry and Trade.

LITERATURE CITED

- Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J. R. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* 284:2148-2152.
- Asai, T., Stone, J. M., Heard, J. E., Kovtun, Y., Yorgey, P., Sheen, J., and Ausubel, F. M. 2000. Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. *Plant Cell* 12:1823-1835.
- Benito, E. P., ten Have, A., van't Klooster, J. W., and van Kan, J. A. L. 1998. Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. *Eur. J. Plant Pathol.* 104:207-220.
- Bestwick, C. S., Brown, I. R., Bennett, M. H. R., and Mansfield, J. W. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell* 9:209-221.
- Bolwell, G. P. 1999. Role of active oxygen species and NO in plant defense responses. *Curr. Opin. Plant Biol.* 2:287-294.
- Collado, I. G., Hernandezgalan, R., Duranpatron, R., and Cantoral, J. M. 1995. Metabolites from a shake culture of *Botrytis cinerea*. *Phytochemistry* 38:647-650.
- Deak, M., Horvath, G. V., Davletova, S., Torok, K., Sass, L., Vass, I., Barna, B., Kiraly, Z., and Dudits, D. 1999. Plants ectopically expressing the iron-binding protein, ferritin, are tolerant to oxidative damage and pathogens. *Nat. Biotechnol.* 17:192-196.
- Deighton, N., Muckenschnabel, I., Colmenares, A. J., Collado, I. G., and Williamson, B. 2001. Botrydial is produced in plant tissues infected by *Botrytis cinerea*. *Phytochemistry* 57:689-692.
- Derckel, J. P., Baillieu, F., Manteau, S., Audran, J. C., Haye, B., Lambert, B., and Legendre, L. 1999. Differential induction of grapevine defenses by two strains of *Botrytis cinerea*. *Phytopathology* 89:197-203.
- Desikan, R., Reynolds, A., Hancock, J. T., and Neill, S. J. 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defense gene expression in *Arabidopsis* suspension cultures. *Biochem. J.* 330:115-120.
- Dickman, M. B., Park, Y. K., Oltersdorf, T., Li, W., Clemente, T., and French, R. 2001. Abrogation of disease development in plants expressing animal antiapoptotic genes. *Proc. Natl. Acad. Sci. USA* 98:6957-6962.
- Duran-Patron, R., Hernandez-Galan, R., Rebordinos, L. G., Cantoral, J. M., and Collado, I. G. 1999. Structure-activity relationships of new phytoxic metabolites with the botryane skeleton from *Botrytis cinerea*. *Tetrahedron* 55:2389-2400.
- Elad, Y. 1997. Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. *Biol. Rev. Cambridge Philosophical Soc.* 72:381-422.
- Gabriel, D. W. 1999. Why do pathogens carry avirulence genes? *Physiol. Mol. Plant Pathol.* 55:205-214.
- Gentile, A. C. 1951. A study of the toxin produced by the isolate of *Botrytis cinerea* from *Exochorda*. *Physiol. Plant* 4:370-386.
- Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10:751-757.
- Govrin, E. M., and Levine, A. 2002. Infection of *Arabidopsis* with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). *Plant Mol. Biol.* 48:267-276.
- Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. 1994. Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. *Cell* 77:551-563.
- Husted, S., and Schjoerring, J. K. 1995. Apoplastic PH and ammonium concentration in leaves of *Brassica napus* L. *Plant Physiol.* 109:1453-1460.
- Isaac, S. 1992. *Fungal-Plant Interactions*. Chapman & Hall, Edinburgh.
- Jennings, D. B., Ehrenshaft, M., Pharr, D. M., and Williamson, J. D. 1998. Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. *Proc. Natl. Acad. Sci. USA* 95:15129-15133.
- Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:251-275.
- Levine, A., Pennell, R., Alvarez, M., Palmer, R., and Lamb, C. J. 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Curr. Biol.* 6:427-437.
- Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:583-593.
- Linthorst, H. J. M. 1991. Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* 10:123-150.
- Lu, H., and Higgins, V. J. 1998. Measurement of active oxygen species generated in planta in response to elicitor AVR9 of *Cladosporium fulvum*. *Physiol. Mol. Plant Pathol.* 52:35-51.
- Mansfield, J. W. 1980. Mechanisms of resistance to *Botrytis*. Pages 181-218 in: *The Biology of Botrytis*. J. R. Coley-Smith, K. Verhoef, and W. R. Jarvis, eds. Academic Press, London.
- Mayama, S., Tani, T., Ueno, T., Midland, S. L., Simms, J. J., and Keen, N. T. 1986. The purification of victorin and its phytoalexin elicitor activity in oat leaves. *Physiol. Mol. Plant Pathol.* 29:1-18.
- Mazel, A., and Levine, A. 2001. Induction of cell death in *Arabidopsis* by superoxide in combination with salicylic acid or with protein synthesis inhibitors. *Free Radic. Biol. Medic.* 30:98-106.
- Miller, J. D., Arteca, R. N., and Pell, E. J. 1999. Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. *Plant Physiol.* 120:1015-1023.
- Mithoefer, A., Daxberger, A., Fromhold-Treu, D., and Ebel, J. 1997. Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. *Phytochemistry* 45:1101-1107.
- Mittler, R., Herr, E. H., Orvar, B. L., van Camp, W., Willekens, H., Inze, D., and Ellis, B. E. 1999. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyperresponsive to pathogen infection. *Proc. Natl. Acad. Sci. USA* 96:14165-14170.
- Murphy, T. M., and Auh, C. K. 1996. The superoxide synthases of plasma membrane preparations from cultured rose cells. *Plant Physiol.* 110:621-629.
- Navarre, D. A., and Wolpert, T. J. 1999. Victorin induction of an apoptotic/senescence-like response in oats. *Plant Cell* 11:237-249.
- Ohashi, Y., and Ohshima, M. 1992. Stress-induced expression of genes for pathogenesis-related proteins in plants. *Plant Cell Physiol.* 33:819-826.
- Pennell, R. I., and Lamb, C. 1997. Programmed cell death in plants. *Plant Cell* 9:1157-1168.
- Pontier, D., Gan, S. S., Amasino, R. M., Roby, D., and Lam, E. 1999. Markers for hypersensitive response and senescence show distinct patterns of expression. *Plant Mol. Biol.* 39:1243-1255.
- Pontier, D., Godiard, L., Marco, Y., and Roby, D. 1994. *Hsr203J*, a tobacco gene whose activation is rapid, highly localized and specific for incompatible plant/pathogen interactions. *Plant J.* 5:507-521.
- Prins, T. W., Tudzynski, P., von Tiedemann, A., Tudzynski, B., Have, A., Hansen, M. E., Tenberge, K., and van Kan, J. A. L. 2000. Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. Pages 33-64 in: *Fungal Pathology*. J. Kronstad, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Rebordinos, L., Cantoral, J. M., Prieto, M. V., Hanson, J. R., and Collado, I. G. 1996. The phytotoxic activity of some metabolites of *Botrytis cinerea*. *Phytochemistry* 42:383-387.
- Reuber, T. L., Plotnikova, J. M., Dewdney, J., Rogers, E. E., Wood, W., and Ausubel, F. M. 1998. Correlation of defense gene induction defects with powdery mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. *Plant J.* 16:473-485.
- Schubert, D., and Chevion, M. 1995. The role of iron in beta-amyloid toxicity. *Biochem. Biophys. Res. Commun.* 216:702-707.
- Siewers, V., Viaud, M., Jimenez-Teja, D., Collado, I. G., Gronover, C. S., Pradier, J. M., Tudzynski, B., and Tudzynski, P. 2005. Functional analysis of the cytochrome P450 monooxygenase gene *bcbot1* of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor. *Mol. Plant-Microbe Interact.* 18:602-612.
- Smith, R. E. 1902. The parasitism of *Botrytis cinerea*. *Bot. Gaz.* 33:421-436.
- Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. 2000. Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) *Arabidopsis* mutants. *Plant Cell* 12:1811-1822.
- Tamagnone, L., Merida, A., Stacey, N., Plaskitt, K., Parr, A., Chang, C. F., Lynn, D., Dow, J. M., Roberts, K., and Martin, C. 1998. Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *Plant Cell* 10:1801-1816.
- Ten Have, A., Mulder, W., Visser, J., and van Kan, J. A. L. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 11:1009-1016.
- Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A., and Broekaert, W. F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* 95:15107-15111.
- Thomma, B., Eggermont, K., Tierens, K., and Broekaert, W. F. 1999. Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiol.* 121:1093-1101.
- Vannell, D., Barbier, M., and Bessis, R. 1991. Study of culture filtrate toxicity of *Botrytis cinerea* observed on grapevine vitroplants. 2. Effect of various fractions isolated from the crude culture filtrate. *Vitis* 30: 213-218.
- Von Caemmerer, S., and Farquhar, G. D. 1981. Some relationships be-

- tween the biochemistry of photosynthesis and the gas exchange of leaves kidney beans. *Planta* 153:376-387.
52. von Tiedemann, A. V. 1997. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* 50:151-166.
 53. Wang, H., Li, J., Bostock, R. M., and Gilchrist, D. G. 1996. Apoptosis: A functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* 8:375-391.
 54. Ward, H. M. 1888. About: Collapse of host cells prior to *Botrytis cinerea* penetration. *Ann. Bot.* 2:319-382.
 55. Wolpert, T. J., Dunkle, L. D., and Ciuffetti, L. M. 2002. Host-selective toxins and avirulence determinants: What's in a name? *Annu. Rev. Phytopathol.* 40:251-285.
 56. Yu, I. C., Parker, J., and Bent, A. F. 1998. Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc. Natl. Acad. Sci. USA* 95:7819-7824.
 57. Zer, H., Peleg, I., and Chevion, M. 1994. The protective effect of desferrioxamine on paraquat-treated pea (*Pisum sativum*). *Physiol. Plant* 92:437-442.
 58. Zipfel, C., and Felix, G. 2005. Plants and animals: A different taste for microbes? *Curr. Opin. Plant Biol.* 8:353-360.