Arabidopsis thaliana: A model host plant to study plant-pathogen interaction using Chilean field isolates of *Botrytis cinerea*

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ABSTRACT

One of the fungal pathogens that causes more agriculture damage is *Botrytis cinerea*. Botrytis is a constant threat to crops because the fungus infects a wide range of host species, both native and cultivated. Furthermore, Botrytis persists on plant debris in and on the soil. Some of the most serious diseases caused by Botrytis include gray mold on vegetables and fruits, such as grapes and strawberries. Botrytis also causes secondary soft rot of fruits and vegetables during storage, transit and at the market.
In many plant-pathogen interactions, resistance often is associated with the deposition of callose, accumulation of autofluorescent compounds, the synthesis and accumulation of salicylic acid as well as pathogenesis-related proteins. *Arabidopsis thaliana* has been used as a plant model to study plant-pathogen interaction. The genome of Arabidopsis has been completely sequenced and this plant serves as a good genetic and molecular model. In this study, we demonstrate that Chilean field isolates infect *Arabidopsis thaliana* and that Arabidopsis subsequently activates several defense response mechanisms associated with a hypersensitive response. Furthermore, we propose that Arabidopsis may be used as a model host species to analyze the diversity associated with infectivity among populations of *Botrytis cinerea* field isolates.

Key terms: *Arabidopsis thaliana, Botrytis cinerea,* plant-pathogen interaction, *PR* genes.

INTRODUCTION

When a plant is infected by a pathogen, one of several possible outcomes can occur. The plant may be unable to control the growth and/or spread of the pathogen, thereby leading to disease and necrosis. Alternatively, the plant may be able to resist pathogen invasion. In some cases, resistance is passive: the plant fails to provide the appropriate environment to support pathogen colonization. In other cases, resistance is an active process in which the plant produces a cascade of defense responses.

Genetic studies of plant-microbe interactions have shown that active resistance usually is conditioned by a plant resistance gene, whose product directly or indirectly recognizes a race-specific avirulence determinant produced by the pathogen (Keen, 1990; Scofield et al., 1996; Tang et al., 1996). One of the most visible signs that a plant is resisting pathogen attack is the development of a hypersensitive response (HR) on the inoculated tissue. The HR is characterized by small necrotic lesions that form around the infection site (Matthews, 1991). These lesions help restrict the growth and spread of the pathogen (Slusarenko et al., 1991). In addition, antimicrobial compounds (phytoalexins) often are produced (Dixon, 1986), and the cell wall is strengthened as a

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result of lignification and cross-linking of cell wall proteins (Bowles, 1990). In addition to HR, the uninfected portion of the plant develops increased resistance to secondary infection by the same as well as unrelated pathogens (Dempsey and Klessig, 1994; Klessig and Malamy, 1994; Ryals et al., 1994; Silva et al., 1999). This non-specific resistance is known as systemic acquired resistance (SAR; Chester, 1933; Ross, 1961). SAR is associated with the systemic expression of SAR-related genes, which include several families of PR genes as well as an increase in the endogenous levels of salicylic acid (SA; Ward et al., 1991; Kombrink and Somssich, 1997).

Various studies have shown that ectopic coexpression of ß-1,3-glucanases (*PR-2*) and chitinases (*PR-3*) in transgenic plants can enhance resistance to phytopathogenic fungi (Broglie et al., 1991; Liu et al., 1994; Yoshikawa et al., 1994; Zhu et al., 1994). Furthermore, constitutive expression of the *PR-1a* gene in tobacco increases resistance to two oomycete pathogens (Alexander et al., 1993). However, constitutively overexpressing *PR* genes does not enhance resistance to all pathogens. Thus although the mechanisms of action of several PR proteins remains to be elucidated, their synthesis serves as a convenient molecular marker for plant disease resistance.

To further characterize the resistance signal transduction pathway, several laboratories have used *Arabidopsis thaliana* as a model organism. *Arabidopsis thaliana* has several major advantages over other species for genetic and molecular studies. It is a small, rapid cycling, self-fertilizing member of the Brassicaceae family (Meyerowitz and Pruitt, 1985; Redei, 1975). Most significantly, it has a small genome (130 Mb) that has been completely sequenced (The Arabidopsis Genome Initiative, 2000). Among the microorganisms that can infect Arabidopsis, fungi are not an exception.

In Chile, *Botrytis cinerea* infects many important crop species, such as grapes, tomatoes, and strawberries. Muñoz et al. (1999, 2002) have identified Chilean field isolates of *Botrytis cinerea* from grapes and tomato. These isolates have been reported to have varying levels of sensitivity or resistance to the fungicide dicarboximide (Muñoz et al., 1999). Additionally, PCR-RFLP marker analysis suggests that isolates sampled from grapes and tomatoes are genetically differentiated. In order to further understand how these different field strains may differentially infect host plants as well as how this may or may not relate to their resistance/susceptibility to the fungicide dicarboximide, we demonstrate in this work, that *Arabidopsis thaliana* may serve as a good model host species to study the interaction between host plants and Chilean field isolates of *Botrytis cinerea*.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) was grown in a mixture of soil-vermiculite (3: 1) in a growth chamber with a 16-h light cycle (140 μE m^{-2} s^{-1}) at 22ºC. Alternatively, seeds were surface sterilized in 0.1% Triton X-100 for 30 minutes, 0.1% Triton X-100 plus 70% ethanol for 5 minutes and 0.1% Triton X-100 plus 2% commercial bleach for 10 minutes, washed 5 times in sterile distilled water, sown on Murashige-Skoog (MS) media containing 0.8% agar and 0.1% sucrose and grown under the same conditions as soil-grown plants.

Fungal maintenance

Botrytis cinerea Chilean grape field isolate U11 (Muñoz et al., 1999) and Chilean tomato field isolate T50 were obtained from Arturo Muñoz (INIA, Carillanca) and grown at 20ºC on potato dextrose agar under continuous light. Spores were collected and suspended in 0.03 M K_2HPO_4, 0.1% glucose and germinated for 3 h at 21ºC.

Fungal infections and fungal growth

Prior to infection, Arabidopsis plants were placed in a growth chamber with a 10-h light cycle (140 μE m^{-2} s^{-1}) at 18ºC and

90% of relative humidity (RH). Upon infection, the relative humidity was increased to 99% by placing the trays containing the plants inside plastic bags. Inoculation with *Botrytis cinerea* was performed on 4-week-old soil-grown plants by depositing 5 µl of a spore suspension (1,000 spores/µl) on a leaf that previously has been punctured with a needle. Alternatively, the plants were infected by spraying them with a Botrytis suspension of 10,000 spores/µl. Infection and analysis of fungal growth were performed as described in Govrin and Levine (2000). The growth of *Botrytis cinerea* was estimated by lactophenol blue staining as describe previously (Govrin and Levine, 2000).

Mock inoculation

Mock inoculation was done by depositing 5 µl of a suspension solution for spores. This solution is composed of 0.03 M K_2HPO_4 and 0.1% glucose (Govrin and Levine, 2000). The same solution was used for mock inoculation when the plants were sprayed.

RNA analyses

Total RNA was extracted from leaves of individual plants using the TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. The final pellet was dissolved in RNase-free water and stored at -80ºC. RNA quality and quantity were determined as described by Meisel et al. (2005). Northern analysis was performed as described by Conrath et al. (1997). Probes specific for the Arabidopsis *PR-1* gene were generated by random primer labeling of an insert of a cDNA clone as described previously (Conrath et al., 1997).

Microscopic analysis of cell-wall associated autofluorescent materials and callose accumulation

Leaves from infected plants were analyzed, at 24 and 48 hrs post infection, for cell-wall-associated autofluorescent material as described by Dempsey et al. (1997) and Silva et al. (1999) and callose accumulation as described by Dietrich et al. (1994). An Olympus IX70 epi-fluorescence microscope with a Sony DXC-390 3CCD color camera with a U-MWU2 filter (Olympus, NY, USA; BP330-385, BA420, DM400) was used for these analyses.

RESULTS

Infection of Arabidopsis with Chilean grape field isolates of Botrytis cinerea

In order to further understand how these different Chilean field strains may differentially infect their host plants, we demonstrate in this work that *Arabidopsis thaliana* may serve as a good model host species to study the interaction between infected plants and native Chilean field isolates of *Botrytis cinerea*.

Chilean grape Botrytis isolate U11 and Chilean tomato Botrytis isolate T50 were used to infect Arabidopsis plants. The plants were infected with varying concentrations of Botrytis (spores/µl) as described in the materials and method section. The susceptibility of *Arabidopsis thaliana* to the grape isolate U11 and the tomato isolate T50 were seen when these Botrytis isolates were placed on the leaf that had been punctured previously with a needle using 5 µl of a 1,000 spores/µl suspension. An Arabidopsis leaf infected with the Chilean isolate U11 is shown in Figure 1A. Within 24 hours after infection with both strains, necrotic lesions formed on the leaf surface at the site of infection (Fig. 1A). However, one week post infection, the Arabidopsis plants appeared more susceptible to the Chilean tomato Botrytis isolate T50, with the infection resulting in larger necrotic lesions than those that formed when Arabidopsis was infected with the Chilean grape Botrytis isolate U11 (data not shown). Infection of the inflorescence stem and the floral apex caused bending and generalized necrosis of the tissue in these regions (Fig. 1B). Furthermore, the hyphae of these fungi were clearly visible as a string-like substance on the surface of these infected plants.

Temporal analysis of the fungal structures upon infection of *Arabidopsis thaliana* with the Chilean grape Botrytis isolate U11 via aniline blue staining indicated that, at 24 h post inoculation, early signs of fungal germination have occurred (Fig. 2A). At 48 h post inoculation, mycelium form (Fig. 2B), and fungal hyphae formed extensively at 72 h post inoculation (Fig. 2C).

Accumulation of callose and phenolic compounds

During the hypersensitive response (HR) triggered by a plant's response to a pathogen attack, physical defenses are activated. One of these physical defenses is the accumulation of callose and phenolic compounds around the entrance site of the pathogen. The accumulation of callose and phenolic compounds are indications that the plant is mounting a defense response against the pathogen. Figure 3 shows that Arabidopsis is mounting a defense response against the Chilean grape Botrytis isolate U11. Infected leaves developed accumulation of callose and phenolics (Fig. 3 A-D), whereas the control leaves did not (Fig. 3 E-H).

Induction of PR-1 gene in the interaction Arabidopsis-Botrytis

It has been shown that when Arabidopsis mounts a resistance response, the *PR* genes are induced. The function of *PR-1* is unknown, but it is considered a good marker for a resistant response (Dempsey et al., 1998). Northern blot analysis reveals an accumulation of *PR-1* following infection with the Chilean grape Botrytis isolate U11 (Fig. 4). *PR-1* transcripts were detected within 24 h post infection. It is interesting to note the differential expression of the *PR-1* gene after the infection by Botrytis. No *PR-1* transcript was detected in mock-inoculated plants.

DISCUSSION

Botrytis cinerea, a necrotrophic fungal pathogen, infects its host plant, resulting in massive tissue destruction and, depending upon the level of infection, may result in plant death. In this paper, we have demonstrated that Chilean *Botrytis cinerea* isolates from grapes (U11) infect *Arabidopsis thaliana*. Fungal spores germinate on the leaves of *Arabidopsis thaliana* within 24 hours. Mycelium are seen within 48 hours and hyphae with 72 hours.

Despite the germination and growth of these fungal spores, our results demonstrate that *Arabidopsis thaliana* has activated several defense strategies to limit or reduce the damage and destruction cause by this necrotrophic fungus. Within 24 hours, we have detected several markers (callose, phenolics compounds, and *PR* genes, see Figures 3 and 4) for the activation of the plant defense system. Several of these markers combined to form what is referred to as a hypersensitive response (HR). HR is characterized by a programmed cell-death process that causes necrosis of the cells surrounding the infection site. Together with this necrosis, there is activation of cell wall lignification, callose deposition and the accumulation of phenolic compounds (Dempsey et al., 1998). Additionally, a well-known marker for HR is the expression of pathogenesis related proteins such as *PR-1*. The HR process has been demonstrated to play an important role in limiting the extent of damage caused by pathogens as diverse as various viral, bacterial and fungal pathogens (Dempsey et al., 1998). Within the same time frame in which we begin to see germination of fungal spores, we have detected accumulation of callose and phenolic compounds in the cells surrounding the infection site. Furthermore, necrotic lesions form at the site of infection, and an increase in *PR-1* transcript levels is detected within this same 24-hour time frame. All of these factors are indicative of a typical hypersensitive defense response. Interestingly, the expression of *PR-1* transcripts decreases at 48 hpi and then increases at least three-fold at 72 hpi. This pattern of expression has been described when the plant needs to sense the pathogen and mount the resistance response, which in

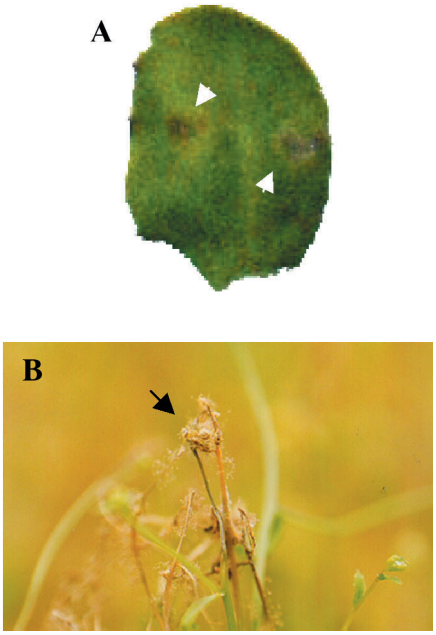

Figure 1: Infection of four-week-old *Arabidopsis thaliana* plants (ecotype Columbia) with *Botrytis cinerea* (Chilean grape isolate U11). A. Lesion formation at the site of inoculation of Chilean grape Botrytis isolate U11 (5 µl of a 1,000 spores/µl suspension). Arrows indicate lesion sites. B. Infection of Arabidopsis plants by spraying (10,000 spores/µl suspension) reveals general necrosis in the inflorescence stem and floral apex (arrow). Note the string-like substance on the surface of these infected plants, indicative of fungal hyphae.

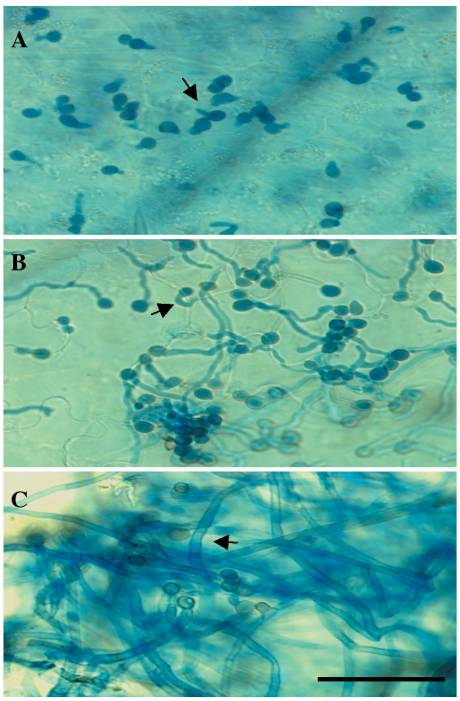

Figure 2: Temporal analysis of the fungal structures upon infection of *Arabidopsis thaliana* with the Chilean grape Botrytis isolate U11 via aniline blue staining. Infected leaf samples were analyzed at 24 h (A), 48 h (B) and 72 h (C) post inoculation, by staining the fungi with aniline blue followed by microscopic analyses. Arrow in A indicates germinating fungi. Arrow in B indicates mycelium. Arrow in C indicates fungal hyphae (Bar represents 30 µm).

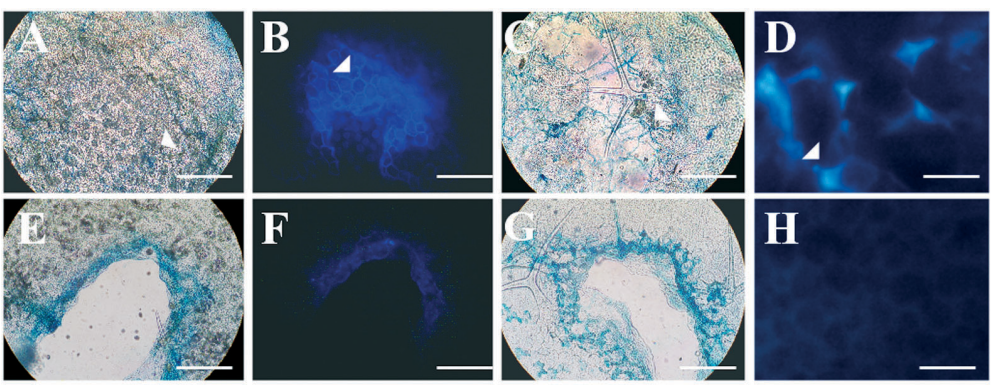

Figure 3: Accumulation of callose and phenolic compounds on Arabidopsis leaves infected with the Chilean grape Botrytis isolate U11. Arabidopsis leaves were infected with the Chilean grape Botrytis isolate U11 (A-D) or mock inoculated as a control (E-H) and analyzed 24 h later. (A and E) Light microscopic analysis of infected/inoculated site. The white arrow shows the spores and mycelium; (B and F) Fluorescence of callose. The arrow shows the accumulation of fluorescence compounds around the cells; (C and G). Aniline blue staining reveals fungal structures. The arrow shows the fungal mycelium; (D and H). Fluorescence of phenolic compounds. The arrows show the accumulation of autofluorescence (the bar in A, B, C, D, E, F and G: 125 µm; in D and H: 50 µm).

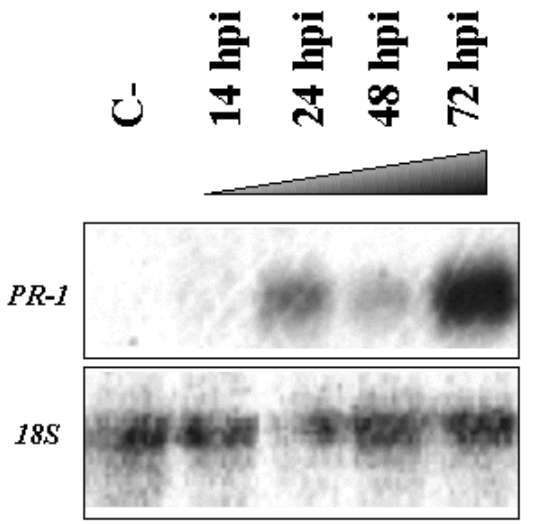

Figure 4: Northern analysis reveals induced expression of *PR-1* in Arabidopsis (ecotype Columbia) leaves infected with the Chilean grape Botrytis isolate U11. C-: negative control (mock inoculated leaf). hpi: hours post infection. 18S: ribosomal RNA as a loading control.

turn triggers a defense response (Govrin and Levine, 2000). Therefore, our results suggest that, although the Chilean Botrytis isolates are able to infect and grow on Arabidopsis, this infection has set off a defense response within the plant.

Still relatively little is know about the plant signal transduction pathways following pathogen infection that lead to the local and systemic activation of defense responses, such as *PR* gene expression. However, numerous studies have demonstrated that SA is an important component of one or more of these pathways. Some of the strongest evidence that SA plays a role in resistance comes from studies on transgenic tobacco and Arabidopsis plants expressing a salicylate hydroxylase-encoding (*nahG*) gene from *Pseudomonas putida*. Salicylate hydroxylase converts SA to cathecol; thus plants expressing the *nahG* gene are unable to accumulate SA at high levels. Strikingly, these *nahG* plants fail to develop SAR and exhibit enhanced susceptibility to pathogen infection (Delaney et al., 1994; Gaffney et al., 1993; Lawton et al., 1995).

We detected an increased lesion size and more robust infection of Arabidopsis when the Chilean tomato Botrytis isolate T50 was used in comparison to the Chilean grape Botrytis isolate (U11) (data not shown). Previous molecular analysis of Chilean Botrytis isolates by Muñoz et al. (1999, 2002) have suggested that there is diversity at the molecular level among Botrytis isolates collected from different species. For example, PCR-RFLP marker analysis showed that isolates sampled from grapes and tomatoes were genetically differentiated (Muñoz et al., 2002). Numerous laboratories have begun characterizing Botrytis isolates at the molecular level (Buttner et al., 1994; Muñoz et al., 2002). Additionally, efforts are underway to sequence the Botrytis genome. However, efforts must be made to establish assays in which the infectivity of these isolates also may be analyzed and associated with the corresponding molecular markers. Therefore, we propose that infection of Arabidopsis with the population of field isolates may serve as a model system to analyze the diversity within the native *Botrytis cinerea* populations. Additionally, this system also may serve to screen the effectiveness of new anti-fungicidal compounds on the infectivity of various Botrytis isolates as well as the effects of these compounds on plants.

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