Botrytis cinerea Manipulates the Antagonistic Effects between Immune Pathways to Promote Disease Development in Tomato

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INTRODUCTION

Plants have evolved sophisticated mechanisms to sense and respond to pathogen attacks. Resistance against necrotrophic pathogens generally requires the activation of the jasmonic acid (JA) signaling pathway, whereas the salicylic acid (SA) signaling pathway is mainly activated against biotrophic pathogens. SA can antagonize JA signaling and vice versa. Here, we report that the necrotrophic pathogen Botrytis cinerea exploits this antagonism as a strategy to cause disease development. We show that B. cinerea produces an exopolysaccharide, which acts as an elicitor of the SA pathway. In turn, the SA pathway antagonizes the JA signaling pathway, thereby allowing the fungus to develop its disease in tomato (Solanum lycopersicum). SA-promoted disease development occurs through Nonexpressed Pathogen Related1. We also show that the JA signaling pathway required for tomato resistance against B. cinerea is mediated by the systemin elicitor. These data highlight a new strategy used by B. cinerea to overcome the plant’s defense system and to spread within the host.
activated by the biotroph suppressed the level of JA-dependent resistance against the necrotroph (Speol et al., 2007). Conversely, JA signaling can act antagonistically on SA-dependent defenses (Glazebrook, 2005). *Pseudomonas syringae* produces the phytotoxin coronatine, which functions as a JA mimic and suppresses SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Brooks et al., 2005; Cui et al., 2005; Glazebrook, 2005; Laurie-Berry et al., 2006). Nevertheless, there are examples of synergistic effects between SA and JA (Glazebrook, 2005; Beckers and Spoel, 2006). Simultaneous activation of SA- and JA-dependent defense pathways resulted in enhanced resistance to pathogenic *P. syringae pv tomato* DC3000 compared with either pathway-related defense response alone (van Wees et al., 2000; O’Donnell et al., 2003). Application of low concentrations of both SA and JA led to enhanced JA response gene expression in the combination treatment compared with JA alone (Mur et al., 2006). Nonexpressed Pathogen Related1 (NPR1) was shown to be a key regulator of SA-mediated suppression of JA signaling (Speol et al., 2003; Ndamukong et al., 2007).

The fungus *Botrytis cinerea* is a plant necrotrophic pathogen that colonizes senescent or dead plant tissues and causes gray mold in vegetables and softening in fruits. Its hyphae can penetrate plant tissues through wounds or natural openings and spread from previously colonized dead tissues into healthy ones (El Oirdi and Bouarab, 2007). *B. cinerea* attacks different plant tissues and has a broad host range of food crops, including tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), grapes (*Vitis vinifera*), and strawberry (*Fragaria spp*), and causes important economic losses, either at pre- or postharvest stages (Coley-Smith et al., 1980). Several virulence factors required for its pathogenicity on different hosts have been previously described (Choquer et al., 2007), including factors involved in phytoalexin detoxification, cell wall–degrading enzymes, toxins, and several genes involved in fungus signaling (Choquer et al., 2007; González-Lamothe et al., 2009; Stefanato et al., 2009).

Successful pathogens have acquired multiple virulence factors to suppress host immunity for their survival. Many pathogens inject a set of effector proteins into host cells through the type III secretion system to interfere with host innate immunity (Hann et al., 2010). These effectors and other virulence factors, including exopolysaccharides, have several targets for suppression, including the hypersensitive response, expression of defense-related genes, cell wall–based defenses, the plant proteasome system, stomatal closure, and the PAMP receptor (Bouarab et al., 2002; Abramovitch and Martin, 2005; Chisholm et al., 2006; Janjusevic et al., 2006; Nomura, 2006; Yun et al., 2006; Rigano et al., 2007; Hann et al., 2010). *P. syringae* manipulates the antagonistic effect between SA and JA to cause disease by producing an analog of JA (Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006). None of the necrotrophic pathogens studied until now are able to activate this antagonism between SA and JA and use it as a strategy to invade or promote their disease in their hosts. These observations led us to investigate whether *B. cinerea* overcomes host immunity and causes disease by manipulating the antagonistic effect between SA and JA signaling pathways. Our findings revealed that *B. cinerea* possesses a virulence factor to circumvent the JA-related defense pathway.

**RESULTS**

**B. cinerea–Tomato Interaction**

Pathogenicity tests were performed on tomato (*S. lycopersicum cv Moneymaker*) leaves inoculated with two isolates of *B. cinerea*, B191 and B8403. Detached leaves from 5-week-old tomato plants were inoculated with mycelium plugs (5-mm diameter) of either isolate. Disease development was analyzed 4 d after inoculation (DAI). Inoculations of tomato leaves with isolate B191 consistently yielded expanding disease lesions (Figure 1A). By contrast, lesion expansion was not observed following inoculation of tomato leaves with the B8403 isolate (Figure 1A). Disease severity was quantified by measuring the size of the necrotic lesions. Isolate B191, but not B8403, induced large necrotic lesions 4 DAI (Figure 1B). We then tested whether the absence of the disease on tomato leaves inoculated with isolate B8403 was
related to the activation of defense responses. Resistance to *Botrytis cinerea* has been known to be JA dependent (Farmer and Ryan, 1992; Li et al., 2003; AbuQamar et al., 2008). Therefore, we examined the expression levels of two JA-dependent genes, *proteinase inhibitors I and II (PI I and PI II)*. Interestingly, the *B. cinerea* isolate B8403 induced high expression levels of JA-dependent genes, PI I and II (Figure 1C). By contrast, the expression of these genes was lower in tomato inoculated with the virulent B191 isolate (Figure 1C). To further investigate the role of JA signaling in resistance against *B. cinerea* isolate B8403, we tested wild-type tomato cv Castlemart and the jasmonate-deficient mutants *def1* (for Defenseless1) and *Spr2* (for suppressor of prosystemin-mediated responses2) (Li et al., 2003). Inoculation with the virulent isolate produced similar responses among wild-type plants and in *def1* and *Spr2* mutants (see Supplemental Figures 1A and 1C online). In contrast with wild-type tomato, *def1* and *Spr2* tomato mutants were susceptible to the *B. cinerea* isolate B8403. The lesion size on leaves of the *def1* and *Spr2* mutants inoculated with isolate B8403 was approximately twofold that of wild-type plants (see Supplemental Figures 1B and 1D online). These results suggest that JA signaling is involved in tomato resistance to *B. cinerea* isolate B8403, which is consistent with previous reports (AbuQamar et al., 2008).

**Systemin Is Involved in Tomato Resistance against *B. cinerea***

The polypeptide systemin is a known elicitor of JA signaling in tomato (Farmer and Ryan, 1992; Li et al., 2003; AbuQamar et al., 2008). We evaluated whether or not systemin is involved in tomato resistance to *B. cinerea*. Quantitative RT-PCR analysis showed that both isolates induced expression of systemin in tomato (Figure 2A). In contrast with the wild type, a systemin antisense tomato line (AS) was susceptible to isolate B8403. The lesion size on leaves of the AS line inoculated with isolate B8403 was approximately fivefold that of wild-type plants (Figure 2B). Figure 2C shows the low level of prosystemin transcripts in AS plants 24 h after inoculation (HAI) with B8403 compared with wild-type plants. Interestingly, *PI I* and *PI II* expression levels induced by isolate B8403 were systemin dependent (Figure 2D), suggesting that resistance of tomato to B8403 is systemin dependent.

**B. cinerea Secretes an Exopolysaccharide That Acts as a Suppressor of the JA Signaling Pathway**

*B. cinerea* has been shown to produce an exopolysaccharide (EPS) known as β-(1,3)(1,6)-D-glucan (Dubourdieu et al., 1981; Stahmann et al., 1995). EPSs are high molecular weight carbohydrates that are produced by several pathogenic fungi and bacteria. They have been established as key virulence factors, and some are involved in suppression of plant immune responses (Yun et al., 2006; Rigano et al., 2007). We purified the EPS from the virulent isolate B191, as described in Methods. The EPS was first observed by atomic force microscopy (AFM; Figure 3A). The results showed that the contour length of the EPS was between 6.5 and 9.67 Å, and the end-to-end distances of the EPS was first observed by atomic force microscopy (AFM; Figure 3A). The results showed that the contour length of the EPS was approximately fivefold that of wild-type plants (Figure 2B).

![Figure 2. Systemin Is Involved in the Interaction between Tomato and *B. cinerea*.](image)

(A) qPCR analysis of systemin gene expression. Five-week-old plants were sprayed with either 10^6 spores/mL of *B. cinerea* B191 or B8403 isolate and then 0, 6, and 12 h later leaves were used to extract RNA. qPCR was performed with specific primers for *tomato prosystemin* and *Actin* (control) as described in Methods and Supplemental Table 2 online. Values represent means ± SD from three biological replicates.

(B) Tomato leaves expressing the antisense of *prosystemin* (AS) are susceptible to the isolate B8403. Detached leaves from 5-week-old wild-type and prosystemin antisense-expressing plants were inoculated with 5-mm-diameter plugs of *B. cinerea* B8403. Photographs were taken (left panel) and lesion size (right panel) analyzed 4 DAI. Error bars represent the SD from three independent experiments (n = 45). Data sets marked with an asterisk are significantly different from inoculated wild-type leaves as assessed by Student’s t test at P < 0.001.

(C) Prosypsystemin expression level in wild-type and AS plants at 0 and 24 HAI with B8403. Values represent means ± SD from three biological replicates.

(D) *PI I* and *PI II* expression levels induced by B8403 are systemin dependent. Five-week-old wild-type and AS plants were sprayed with 10^6 spores/mL of *B. cinerea* B8403 isolate, and then 48 h later leaves were used to extract RNA. Expression levels of *PI I* and *PI II* relative to *Actin* were measured by qPCR with specific primers for *PI I* and *PI II* as described in Methods and Supplemental Table 2 online. Values represent means ± SD from three biological replicates.
Figure 3. *B. cinerea* Secretes a β-(1,3)(1,6) glucan (EPS).

Characterization of the EPS produced by *B. cinerea*.

(A) AFM image of the pure EPS.

(B) TLC of the hydrolyzed EPS (HEPS). Man, Gal, and Glc were used as standards.

(C) Gas chromatography–mass spectrometry profiles of standards shown in (B).

(D) The sugar derivatives from the hydrolyzed EPS.

(E) Heteronuclear 1H–13C chemical shift correlated spectrum of the EPS.

(F) 13C and 1H NMR assignments of the EPS. Letters in parentheses refer to the glucopyranose units shown in (G).

(G) A possible repeating unit for the EPS produced by *B. cinerea*. A, B, and C refers to letters in parentheses shown in (F).

(H) Quantification of the EPS amount (in μg per mg dry weight) produced in PDB medium by B191 and B8403 isolates. Error bars represent the SD (*n* = 3). The experiment was repeated at least three times with similar results.

[See online article for color version of this figure.]
molecules was between 0.9 and 1.26 μm, demonstrating that the EPS is a high molecular weight polysaccharide (Figure 3A). To determine the nature of this EPS, acid hydrolysis was performed and the products were analyzed by thin layer chromatography (TLC) and gas chromatography (GC). Pure Glc, Gal, and manose were used as controls. TLC and GC experiments showed that the main monomeric constituent of the EPS was Glc (Figures 3B to 3D). Glycosidic linkages were determined by two-dimensional nuclear magnetic resonance analysis (Figure 3E). The carbon-13 heteronuclear multiple quantum coherence analysis showed signals indicating the presence of a β-(1-3)(1,6)-glucan backbone, namely, 102.88 ppm (C-1), 73.64 ppm (C-2), 87.09 ppm (C-3), 69.91 ppm (C-4), 76.67 ppm (C-5), and 60.84 ppm (C-6). C-1 signals at low field and H-1 signals at high field indicated a β-configuration. There was an additional signal corresponding to anomeric carbon at 102.92 ppm (Figure 3F). This, together with the downfield displacement of the signal corresponding to C-6 at 68.56 ppm, indicates some degree of substitution at the C-6 position by β-D-glucopyranosyl stubs on the main backbone (Figures 3B to 3F). Signals at 72.42, 76.76, and 60.86 ppm can be attributed to C-2, C-3, and unsubstituted C-6, respectively, of the aforementioned six-linked stubs. These data suggest that the EPS produced by isolate B191 corresponded to the β-(1,3)(1,6) glucan that has been described previously (Dubourdieu et al., 1981; Stahmann et al., 1995) (Figure 3G). Interestingly, we found that isolate B191, which causes disease in tomato, produces large quantities of the EPS, whereas isolate B8403 produces much lower amounts (Figure 3H). These results led us to investigate the role of this glucan in the disease caused by B. cinerea. Tomato leaves were sprayed with 50 μg/mL of purified EPS 24 h before inoculation with either B. cinerea isolate, B191 or B8403 (Figure 4). Remarkably, tomato leaves pretreated with EPS showed disease symptoms in response to isolate B8403, whereas mock-treated plants were still resistant to B8403 (Figure 4A). The size of lesions recovered from B8403-inoculated leaves of tomato plants that had been pretreated with the EPS was approximately twofold larger than that of the mock-treated plants (Figure 4B). The symptoms and necrotic lesions induced by the virulent isolate B191 in plants pretreated with EPS were similar to the mock-treated ones (Figures 4A and 4B). We then examined five other B. cinerea isolates described in Supplemental Figure 1 online for EPS production and lesion development on S. lycopersicum cv Moneymaker as described below. Results show that the lesion areas caused by B. cinerea isolates are correlated with the concentration of EPS that they produced (see Supplemental Figures 2A and 2B online). This suggests that EPS plays an important role in the virulence of B. cinerea.

As several EPSs are plant defense suppressors (Yun et al., 2006; Rigano et al., 2007), we then investigated if β-(1,3)(1,6)-D-glucan suppresses JA-dependent defense markers (PI I and II) induced by isolate B8403 in tomato. Five-week-old tomato plants were pretreated with either water or EPS 24 h before inoculation, which was performed by spraying the plants with spores (10⁶ spores/mL) of either isolate. The plants were then incubated in a high-humidity growth chamber, with samples harvested at 0, 24, and 48 HAI. Transcript levels of PI I and II were detected by quantitative PCR (qPCR). Significant reduction in PI I and PI II expression was observed in inoculated leaves pretreated with EPS compared with those pretreated with water (Figure 4C). We conclude that the EPS permits B. cinerea growth in tomato by compromising JA-dependent defenses. The minimal concentration of EPS required to restore disease symptoms was 25 μg/mL, and the minimum time between EPS treatment and inoculation that was required to observe the suppression effect was 12 h (see Supplemental Figures 3A and 3B online). We estimated the level of EPS produced by a 48-h culture in potato dextrose broth (PDB) medium of isolate B191 to be 160 μg/mL. This suggests that the concentration of the EPS used in this study was physiologically relevant.

B. cinerea Manipulates, through Its EPS, the Antagonism between SA and JA to Promote Disease in Tomato

β-Glucans are also known to act as elicitors of plant immune responses, including SA-dependent defense (Klarzynski et al., 2000). Since isolate B191 produced large quantities of the EPS, we then tested whether or not this fungus and its EPS induce SA accumulation in tomato. Five-week-old tomato plants were sprayed with water or with 10⁶ spores/mL of either isolate B191 or B8403. The accumulation of SA was quantified by HPLC 12 and 24 HAI. By contrast, 5-week-old tomato plants were sprayed with either water or β-(1,3)(1,6)-D-glucan (50 μg/mL), and samples were harvested 3 and 6 h after treatment for SA quantification. Our experiments showed that, in contrast with water-treated and B8403-inoculated tomato plants, those inoculated with isolate B191 and treated with the EPS accumulated SA (Figure 5A).

Here, we have shown that EPS induces tomato susceptibility to B. cinerea, suppresses expression of the JA-dependent genes PI I and PI II, and induces the accumulation of SA (Figures 4 and 5). As it has been established that SA can antagonize JA, we then tested if SA itself induces susceptibility of tomato to the B8403 isolate. Five-week-old tomato plants were watered and sprayed with 0.05, 0.5, or 2.5 mM of SA, and 24 h afterwards, detached leaves were inoculated with mycelium plugs of isolate B8403. As shown in Figure 5B, plants pretreated with SA became susceptible to B8403, and this susceptibility varied with the SA concentration. The lesions on SA-pretreated leaves inoculated with B8403 were significantly larger than those on water-treated leaves (Figure 5C). Interestingly, a significant reduction in PI I and PI II expression was observed in B8403-inoculated leaves that had been pretreated with SA compared with those pretreated with buffer (Figure 5D). SA-deficient NahG plants (Brading et al., 2000) were also less susceptible to B. cinerea (Figure 6). In addition, EPS-induced tomato susceptibility to B. cinerea was affected in NahG plants, suggesting that the EPS effect occurred through the SA pathway (Figures 6B and 6C). We then tested whether EPS enhances susceptibility of JA-deficient mutants def1 and spr2 to B. cinerea isolate 8403. Plants were pretreated with EPS (50 μg/mL), and 24 h later detached leaves were inoculated with isolate B8403 as described below. EPS did not enhance susceptibility of def1 or spr2 mutants to B. cinerea compared with mock treatment (see Supplemental Figure 4 online). These results demonstrate that B. cinerea uses its EPS to manipulate the antagonistic effect between SA and JA to enhance gray mold expression in tomato.
Figure 4. β-(1,3)(1,6)-D-Glucan Induces Susceptibility of Tomato to B. cinerea by Suppressing JA Signaling.

(A) and (B) Inoculation of tomato leaves with B. cinerea isolates B191 and B8403. Five-week-old tomato plants were sprayed with water or EPS; 24 h later, detached leaves were inoculated with 5-mm-diameter plugs of either B. cinerea B8403 or B191 isolate. Photographs were taken (A) and lesion size was analyzed (B) 4 DAI. Error bars represent the SD from three independent experiments (n = 45). Data sets marked with an asterisk are significantly different from inoculated mock-treated plant leaves according to Student’s t test at P < 0.001.

(C) Expression levels of tomato PI I and PI II relative to Actin. Five-week-old plants were sprayed with water or EPS; 24 h after treatment, tomato plants were sprayed with either 10⁶ spores/mL of B. cinerea B191 or B8403 isolate or water; 0, 24, and 48 HAI, samples were harvested for RNA extraction. qPCR was performed with specific primers for tomato PI I, PI II, and Actin (control) as described in Methods and Supplemental Table 2 online. Values represent means ± SD from three biological replicates.
As the EPS induces accumulation of SA in tomato (Figure 5), we tested if it confers resistance against the hemibiotrophic pathogen *P. syringae* DC3000. Interestingly, three days after inoculation with virulent *P. syringae* DC3000, plants pretreated with EPS displayed an eightfold lower bacterial titer compared with the mock-pretreated plants, confirming that the EPS induces the SA signaling pathway (see Supplemental Figure 5 online).

To investigate whether both isolates regulate the JA signaling pathway up- or downstream of JA synthesis, the accumulation of JA was assessed using ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS). Tomato plants
were sprayed with water or 10^6 spores/mL of either isolate B191 or B8403. Samples were harvested for JA quantification at 0, 12, and 24 HAI. Both isolates induced the accumulation of JA to levels that are about ninefold that of the mock-treated plants (Figure 7). These results suggest that the suppression of JA-dependent PI I and PI II expression induced by the virulent isolate B191 but not B8403 is mainly downstream of the JA synthesis pathway.

**NPR1 Promotes Disease Caused by B. cinerea in Tomato**

It is well known that the SA effect occurs mainly through the coactivator NPR1 (Durrant and Dong, 2004; Pieterse and Van Loon, 2004). These observations led us to test whether NPR1 promotes disease caused by *B. cinerea*. We first checked if *B. cinerea* regulates the expression of NPR1 in tomato. Five-week-old tomato plants were sprayed with spores (10^6 spores/mL) of either isolate. Plants were then incubated in a growth chamber with high humidity, and the samples were harvested at 0, 6, and 12 HAI. The expression of NPR1 was evaluated by qRT-PCR. Interestingly, the expression level of NPR1 is much higher in tomato inoculated with isolate B191 than with isolate B8403 at 12 HAI (Figure 8A). To test whether this regulation has a biological significance in the interaction between *B. cinerea* and tomato, virus-induced gene silencing (VIGS) experiments were performed, in which tomato plants were inoculated with a tobacco rattle virus (TRV) vector containing 300 bp of the cDNA of tomato NPR1 (TRV:NPR1) (Ratcliffe et al., 2001; El Oirdi and Bouarab, 2007). Control plants were inoculated with the TRV vector without the cDNA insert (TRV:00). After 3 weeks, the level of NPR1 transcripts was analyzed in silenced plants by qRT-PCR. TRV:NPR1 silenced plants showed a significantly lower level of NPR1 compared with TRV:00-silenced plants (Figure 8B). Detached leaves from TRV-infected plants were then challenged with plugs of B191, the virulent isolate of *B. cinerea*. The disease was scored by the surface of the necrotic area 4 DAI. TRV:00-inoculated plants that had subsequently been challenged with plugs of the isolate B191 showed disease symptoms resembling those produced in wild-type plants (Figures 1A and 8C). Interestingly, the necrotic area was significantly reduced on leaves of NPR1-silenced plants compared with leaves from TRV:00-silenced ones (Figures 8C and 8D). Tomato is resistant to B8403, which explains why we did not test the infection of B8403 in

**Figure 6.** EPS-Induced Tomato Susceptibility to *B. cinerea* Occurs through SA.

(A) Detached leaves from wild-type or SA-deficient NahG plants were inoculated with 5-mm-diameter plugs of either *B. cinerea* B8403 or B191 isolate, and disease size was analyzed 4 DAI.

(B) and (C) Five-week-old tomato wild-type and NahG plants were sprayed with water or EPS (50 μg/mL); 24 h after detached leaves were inoculated with 5-mm-diameter plugs of either *B. cinerea* B191 (B) or B8403 (C) isolate and disease size were analyzed 4 DAI. Error bars represent the SD from three independent experiments (n = 45). Within each panel, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences at P < 0.001.

were sprayed with water or 10^6 spores/mL of either isolate B191 or B8403. Samples were harvested for JA quantification at 0, 12, and 24 HAI. Both isolates induced the accumulation of JA to levels that are about ninefold that of the mock-treated plants (Figure 7). These results suggest that the suppression of JA-dependent PI I and PI II expression induced by the virulent isolate B191 but not B8403 is mainly downstream of the JA synthesis pathway.

**Figure 7.** JA Accumulation in Response to *B. cinerea* Inoculation.

Quantification of JA after *B. cinerea* inoculation. Tomato plants were sprayed with water or with 10^6 spores/mL of isolates B191 or B8403. The accumulation of JA was quantified using UPLC-MS/MS, 0, 12, and 24 HAI. The data presented are the means of three biological replicates, and error bars represent the SD.
Figure 8. NPR1 Induces Disease Development caused by B. cinerea in Tomato

(A) Expression pattern of tomato NPR1 transcripts after B. cinerea infection. Five-week-old plants were sprayed with either 10⁶ spores/mL of B. cinerea B191 or B8403 isolate; 0, 6 and 12 HAI, samples were harvested for RNA extraction. qRT-PCR was performed with specific primers for tomato NPR1 and Actin (control) as described in Methods and Supplemental Table 2 online.

(B) NPR1 transcript level in silenced plants. VIGS was performed using a TRV vector. Seedlings were inoculated with TRV:Sl NPR1 or empty vector as a control (TRV:00). Three weeks after inoculation, RNA was isolated and qRT-PCR was performed as below. Error bars represent the SD from three biological replicates.

(C) and (D) Tomato seedlings were silenced with the indicated TRV construct as described in (B). Three weeks later, detached leaves were infected with B. cinerea B191. Photographs were taken (C) and disease area was analyzed (D) 4 DAI. Error bars represent the SD (n = 60) from three independent experiments. Data sets marked with an asterisk are significantly different from TRV:00-infected leaves as assessed by Student’s t test at P < 0.001.

(E) to (G) Overexpression of Arabidopsis NPR1 enhances susceptibility to B191 isolate and compromises resistance to B8403 in tomato.

(E) Expression levels of Sl NPR1 and At NPR1 transcripts in wild-type and transgenic tomato plants (S. esculentum line CL5915-93D4-1-0-3) expressing Arabidopsis NPR1. Actin was used as a control.

(F) and (G) Detached leaves from 5-week-old wild-type and transgenic tomato plants (S. esculentum line CL5915-93D4-1-0-3) expressing the Arabidopsis NPR1 were infected with either B. cinerea B191 or B8403. Photographs were taken [(F), left panel for B191 and right panel for B8403] and disease area was analyzed, 4 DAI [(G), left panel for B191 and right panel for B8403]. Error bars represent the SD (n = 60) from three independent experiments. Data sets marked with an asterisk are significantly different from wild-type infected leaves as assessed by Student’s t test at P < 0.01.
NPR1-silenced plants. These results suggest that *B. cinerea* uses NPR1 to enhance its disease in tomato leaves. Transgenic tomato plants (*S. lycopersicum* line CL5915-93D4-1-0-3) over-expressing *Arabidopsis NPR1* have been described previously (Lin et al., 2004). We used those plants to test whether the overexpression of NPR1 enhances disease caused by *B. cinerea*. The levels of tomato and *Arabidopsis NPR1* transcript in the transgenic and wild-type tomato plants were confirmed by RT-PCR as described previously (Lin et al., 2004; Figure 8E). Then, detached leaves from 5-week-old wild-type and transgenic plants were infected with mycelium plugs of both isolates. Leaves from plants overexpressing *Arabidopsis NPR1* were more susceptible to the virulent isolate (B191) than those from the wild-type plants (Figures 8F and 8G). Furthermore, leaves from transgenic plants were susceptible to the isolate B8403 (Figures 8F and 8G). These data suggest that NPR1 promotes the disease development caused by *B. cinerea* in tomato.

We showed that SA and EPS promote disease development caused the B8403 isolate of *B. cinerea* in tomato (Figures 4 and 5). To investigate whether SA-enhanced susceptibility to *B. cinerea* occurs through NPR1, we sprayed TRV:00- and TRV: NPR1-silenced plants with SA (0.5 mM) or EPS (50 μg/mL), and 24 h later detached leaves were inoculated with plugs of B8403 isolate. As controls, TRV-infected plants were sprayed with the buffer and water that was used to dissolve SA and EPS, respectively. As expected, B8403 was not able to infect leaves from TRV:00 plants, and infection was significantly higher after pretreatment with either SA or EPS compared with mock-treated plants (Figures 9A and 9B). Remarkably, neither SA nor EPS were able to induce susceptibility to B8403 in TRV:00-silenced plants (Figures 9A and 9B). These results suggest that disease promoted by either SA or EPS occurs through NPR1.

We extended our experiments to ask whether NPR1 regulates the expression of *PI I* and *PI II*. TRV:00- and TRV: NPR1-silenced plants were sprayed with 10^6 spores of *B. cinerea* isolate B191, and samples were harvested for RNA extraction at 0, 24, and 48 hAI. The effects of NPR1 knockdown on *PI I* and *PI II* expression were analyzed by qRT-PCR. In contrast with TRV:00, activation of both *PI I* and *PI II* was already observed in the absence of any inoculation in NPR1 knockdown plants (Figure 10). The expression of both *PI I* and *PI II* genes was significantly induced in NPR1 knockdown in response to B191 inoculation compared with the control (TRV:00) (Figure 10). These results suggest that NPR1 suppresses the expression of the JA-dependent genes *PI I* and *PI II*.

**PI I and PI II Are Required for Resistance against B. cinerea in Tomato**

We show below that *B. cinerea* suppresses the expression of *PI I* and *PI II* through NPR1. We then investigated whether *PI I* and *PI II* are required for tomato resistance against *B. cinerea*. TRV:00-, TRV:PI I–, and TRV:PI II–silenced plants were generated, and the level of *PI I* and *PI II* was analyzed by qRT-PCR. The results showed that the transcript levels of these genes were lower in TRV:PI I– and TRV:PI II–silenced plants than in TRV:00 plants (Figure 11A). The silencing of *PI I* did not affect the expression of *PI II* and vice versa (Figure 11A). Detached leaves were then inoculated with plugs of either *B. cinerea* B191 or B8403. TRV:PI I– and TRV:PI II–silenced plants were more susceptible than TRV:00-silenced plants to either B191 or B8403 (Figure 11B). These results suggest that *PI I* and *PI II* are important for tomato resistance against *B. cinerea*.

**DISCUSSION**

The intimate relationship between eukaryotes and microbial pathogens has led to the coevolution of a number of complex strategies for attack and defense. Plants have evolved a powerful and multilayered defense system to fight infection by most microbial organisms. It has become clear that different defense pathways are differentially effective against specific types of invaders. In general, biotrophic pathogens that depend entirely on live host cells for their nutrient supply are more sensitive to SA-dependent responses, whereas necrotrophic pathogens that benefit from host cell death usually are better controlled by
Plants show high susceptibility to *Botrytis cinerea* (2005). SA-deficient NahG tomato plants are more susceptible to appear to play a major role (Ferrari et al., 2003; Glazebrook, and Spoel, 2006; Mur et al., 2006; Koornneef et al., 2008; Koornneef and Pieterse, 2008). Crosstalk between SA and JA presumably reduces fitness costs of inappropriate resistance and provides the plant with a regulatory potential to fine-tune the defense reaction based on the nature of the pathogen. *P. syringae* produces the phytotoxin coronatine, which functions as a JA analog. During the interaction with susceptible *Arabidopsis* plants, coronatine suppresses SA-dependent defenses, thereby promoting susceptibility to this pathogen (Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006). Silverleaf whitely induces SA-based responses and suppresses otherwise effective JA defenses. However, the mechanism by which this SA is accumulated is still unknown. (Zarate et al., 2007). In addition to their role in local resistance, SA and JA are also important for systemic resistance (Shah, 2009). It is then possible that the antagonisms activated by the EPS may have an effect on systemic resistance against *B. cinerea* and other pathogens. It has been shown that infection with hemibiotrophic *P. syringae*, which induces SA-mediated defense, rendered plants more susceptible to the necrotrophic pathogen *Alternaria brassicicola* by suppressing the JA signaling pathway. This process was partly dependent on the crosstalk modulator NPR1. This tradeoff was restricted to tissues adjacent to the site of initial infection (Spoel et al., 2007). It has not been previously demonstrated that a necrotrophic pathogen can itself activate this antagonism as a strategy to cause disease. Our results show that *B. cinerea* produces an EPS that activates the SA pathway. In turn, the latter antagonizes the JA signaling pathway through NPR1, thereby allowing the fungus to enhance its disease in tomato (Figure 12). In addition to the EPS, several other virulence factors have been described previously, such as phytotoxins and lytic enzymes (Choquer et al., 2007; González-Lamothé et al., 2009). It is possible that those virulence factors contribute to the success of the antagonism between SA and JA mediated by the EPS. Soil bacteria belonging to various genera of the order *Rhizobiales* (collectively called rhizobia) are able to invade leghume roots in nitrogen-limiting environments, leading to the formation of a highly specialized organ, the root nodule (Soto et al., 2006; Jones et al., 2007). A bacterial EPS is required for the establishment of the nitrogen-fixing symbiosis between *Rhizobium meliloti* and its host plant alfalfa (*Medicago sativa*). NRPs are involved in nodule invasion by *Sinorhizobium meliloti* (Niehaus et al., 1993; Hoang et al., 2004; Jones et al., 2008). On the other hand, some EPSs from *S. meliloti* suppress defense responses to optimize the symbiosis success (Niehaus et al., 1993; Hoang et al., 2004; Jones et al., 2008). Our results show that *B. cinerea* induces, through its EPS, the accumulation of SA, which suppresses the JA signaling pathway downstream of JA synthesis. Doares et al. (1995) provided evidence that exogenous application of SA to tomato plants strongly inhibits the JA-induced expression of genes encoding PI I and II, suggesting that SA targets the JA pathway downstream of JA biosynthesis. Recently, Leon-Reyes et al. (2010) showed that SA-mediated suppression of JA-responsive gene expression in *Arabidopsis* is targeted downstream of the jasmonate biosynthesis pathway. NPR1 and the TGA (for thymine, guanine, and adenine nucleotides) binding sequence protein family are important for the SA signaling pathway (Loake and Grant, 2007).

JA-dependent defense (Glazebrook, 2005). The role of SA signaling in plant resistance to *B. cinerea* is complex. Although it contributes to resistance of *Arabidopsis* against *B. cinerea*, it does not appear to play a major role (Ferrari et al., 2003; Glazebrook, 2005). SA-deficient NahG tomato plants are more susceptible to *B. cinerea* than are wild-type plants (Figure 6). SA-deficient NahG *Arabidopsis* plants show high susceptibility to *B. cinerea*; however, SA-deficient NahG *Nicotiana benthamiana* plants react similarly as the wild type to *B. cinerea* (Govrin and Levine, 2002; Ferrari et al., 2003; Asai et al., 2010). It is possible that the antagonism between SA and JA activated by *B. cinerea* is strain and host dependent. It might also depend on the coevolution between strains of *B. cinerea* and their hosts. Using JA-deficient mutants, it was shown recently that additional pathways modulate *Arabidopsis*–*B. cinerea* interactions (Rowe et al., 2010). Pharmacological and genetic experiments revealed that SA- and JA-dependent pathways are reciprocally antagonistic (Beckers and Spoel, 2006; Mur et al., 2006; Koornneef et al., 2008; Koornneef and Pieterse, 2008). This antagonism has been shown to be SA and JA dose dependent (Beckers and Spoel, 2006; Mur et al., 2006; Koornneef et al., 2008; Koornneef and Pieterse, 2008).

**Figure 10.** NPR1 Negatively Modulates the Expression of PI I and PI II.

Expression levels of PI I and PI II after inoculation of NPR1 knockdown tomato plants with *B. cinerea* isolate B191. TRV:00- and TRV:SI NPR1-silenced plants were sprayed with 10⁶ spores/mL of *B. cinerea* B191; samples were harvested for RNA extraction 0, 24, and 48 HAI. qRT-PCR was performed as described in Methods and Supplemental Table 2 online. Error bars represent the SD from three biological replicates.
These two regulators likely play a role in the antagonism between SA and JA (Spoel et al., 2003; Yuan et al., 2007). It was shown recently that ethylene, another plant hormone, modulates the antagonism between SA and JA that is mediated by NPR1 (Leon-Reyes et al., 2009). Overexpression of the SA-regulated glutaredoxin GRX480 was found to antagonize JA-responsive PDF1.2 transcription; this suppression requires NPR1 and TGA transcription factors (Ndamukong et al., 2007). Here, we show that *B. cinerea* manipulates NPR1 to establish the antagonism between SA and JA signaling, thus enhancing tomato disease. We also show that systemin is involved in resistance of tomato to *B. cinerea*. Systemin is an 18–amino acid peptide that is derived from a 200–amino acid precursor called prosystemin, which is released from the wound site and triggers defense responses (Ryan and Pearce, 2003). Systemin has been shown to accumulate in members of the solanaceae family, including tomato, potato, pepper (*Capsicum annuum*), and tobacco (*Nicotiana tabacum*; Constabel et al., 1998; Ryan and Pearce, 2003; Pearce et al., 2009; Heiling et al., 2010).

The ability of *B. cinerea* to manipulate the antagonistic effect between SA and JA pathways, through the EPS elicitor-induced SA, distinguishes it from all other fungi, which, as far as we are aware, have not been reported to activate this antagonism and use it as a strategy to infect their hosts. It will be of interest to examine whether other necrotrophic pathogens are able to exploit this antagonism between SA and JA to cause or enhance plant diseases.

It is possible that other hormones are involved in the manipulation mediated by *B. cinerea* EPS. SA and JA do not act independently to confer resistance against pathogens but operate in complex networks with crosstalk to several other phytohormonal signaling pathways (Robert-Seilaniantz et al., 2007; Spoel and Dong, 2008; Grant and Jones, 2009; Verhage et al., 2010). Abscisic acid (ABA) influences many plant–pathogen interactions depending on pathogen’s lifestyle. ABA-deficient plants are more resistant to *B. cinerea* (AbuQamar et al., 2006; Asselbergh et al., 2007; Curvers et al., 2010). However, Arabidopsis ABA-deficient mutants are hypersensitive to the oomycete *Pythium irregulare* and the fungus *Leptosphaeria maculans* (Adie et al., 2007; Kaliff et al., 2007). *P. syringae* pv tomato hijacks the Arabidopsis ABA signaling pathway to cause disease (de Torres-Zabala et al., 2007). ABA can suppress the callose deposition induced by the PAMP flg22, a peptide derived from the bacterial PAMP flagellin (de Torres-Zabala et al., 2007; Clay et al., 2009). It has been shown that SA represses auxin signaling, and, inversely, auxin treatments decrease the SA response (Wang et al., 2007). Flg22 induces the expression of microRNA (miR393), which targets the auxin receptor F-box protein TIR1 and its paralogs AFB2 and AFB3. Plants that overexpress miR393 are more resistant to *P. syringae* pv tomato (Navarro et al., 2006). On the other hand, it has been proposed that auxin signaling and transport are essential to establish systemic acquired resistance, although the molecule/s involved in this mechanism could be indole-3-acetic acid and/or other indolic derivatives (Truman et al., 2010). DELLA proteins, plant growth repressors whose degradation is promoted by the phytohormone gibberellin (Sun and Gubler, 2004), regulate plant immune...
response by modulating the balance of JA and SA signaling (Navarro et al., 2008).

Our data present an important advance in our understanding of the strategies used by pathogens to manipulate their hosts. An exciting future challenge will be the biochemical and genetic elucidation of this suppression effect triggered by the EPS, which may have implications for our understanding of the strategies used by necrotrophic pathogens to overcome plant defenses and subsequently establish disease. This is a prerequisite for further development of a solid knowledge-based integrated strategy toward management of diseases caused by necrotrophic pathogens.

METHODS

Pathogen Growth Conditions

The Botrytis cinerea wild-type isolates B191, B8403, 10-0364, 2026, and B87 that were used in this study were provided by the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada) and MAPAQ (Quebec Ministry of Agriculture, Fisheries, and Food). These isolates were subcultured as previously described (El Oirdi et al., 2007; El Oirdi et al., 2010).

Plant Material

Solanum lycopersicum cv Moneymaker, S. lycopersicum overexpressing Arabidopsis thaliana NPR1 and its wild-type line CLS915-93D4-1-0-3, S. lycopersicum cv Castlemart cultivars, and Def1, prosystemin antisense, and Spr2 mutants were grown on soil in a growth chamber at 60% relative humidity and under a long-day photoperiod consisting of a 16-h light regime with a photosynthetic photon flux density of 150 μmol photons m⁻² s⁻¹ at 23 °C, followed by an 8-h dark period at 18 °C.

Plant Inoculations

Detached leaves from 5-week-old tomato plants were inoculated as previously described (El Oirdi and Bouarab, 2007; El Oirdi et al., 2010).

For glucan treatments, leaves of 5-week-old plants were sprayed with purified glucan (50 μg/mL) containing 0.02% Silwet L-77 to reduce surface tension. For SA treatments, plants were watered with 0.05, 0.5, or 2.5 mM SA (Sigma-Aldrich) in 20 mM sodium phosphate buffer, pH 6.5, and sprayed with the same solution containing 0.02% Silwet L-77. Control plants were watered with 20 mM sodium phosphate buffer, pH 6.5, and sprayed with this buffer containing 0.02% Silwet L-77. Twenty-four hours later, detached leaves from mock-, SA-, and glucan-pretreated plants were inoculated with B. cinerea as previously described (El Oirdi and Bouarab, 2007; El Oirdi et al., 2010). Disease lesion sizes, which correspond to the area of the lesion (mm²), were made using AxioVision software (version 4.6.3, 2007). For qRT-PCR experiments, the inoculation tests were performed in plants; 5-week-old plants were sprayed with the spores (10⁶ spores/mL) of B. cinerea isolates B191 or B8403. The plants were then incubated in a growth chamber with high humidity. The same experiment was done with mock-, SA-, and glucan-pretreated plants. The samples were then harvested for RNA extraction.

For bacterial growth, tomato plants were vacuum infiltrated with Pseudomonas syringae DC3000 (~0.01 OD) suspended in 10 mM MgCl₂ and 0.02% Silwet. Bacterial leaf populations were measured from three leaves per plant and three plants per treatment, 3 DAI.

TRV-Based VIGS in S. lycopersicum

A 300-bp fragment from tomato NPR1, TGA1a, PI I, and PI II cDNAs was PCR amplified using the primers described in Supplemental Table 2 online. The fragments were cloned in the Smal site of the pTRV-RNA2 vector (Ratcliff et al., 2001; Liu et al., 2002). As a control, the pTRV-RNA2 empty vector was used (TRV:00). Agrobacterium tumefaciens cultures (o.d. 1) containing either TRV:00 or TRV:NPR1 were mixed with those containing pTRV-RNA1 (o.d. 1) in a 1:1 ratio before agroinfiltration. S. lycopersicum seedlings were infiltrated with the mixture, and, 3 weeks later, the level of targeted transcripts was analyzed by qRT-PCR using the primers described in Supplemental Table 2 online.

Characterization of B. cinerea Extracellular β-(1,3)(1,6)-D-Glucan (EPS)

Isolates B191 or B8403 (10⁶ spores/mL) were cultivated in 80 mL of PDB medium, and the EPS was purified and quantified at 0, 24, 48, 72, and 96 HAI as previously described (Dubourdieu et al., 1981; Stahmann et al., 1995). The major peak was pooled, lyophilized, and resuspended in water for further analysis. The EPS was quantified 72 HAI for 10-0364, 2026, 2029, and B87 isolates.

Carbohydrate analysis of the EPS by TLC was performed after hydrolysis of the sample in 1 M HCl for 4 h at 100 °C. The hydrolyzed sample was subjected to TLC on silica gel G in butan-1-ol/ethanol/water (5:5:4 v/v/v) with three developments. Carbohydrates were detected by spraying the plate with a solution of 5% (v/v) sulphuric acid in ethanol, followed by heating at 120 °C.

Monosaccharide composition of EPS was determined by GC after hydrolysis in 2 M TFA at 121 °C for 4 h and further derivatization to alditol acetates. The hydrolyzed samples were then injected into a Hewlett-Packard 5890A GC equipped with a flame ionization detector and fitted with a fused-silica column (0.25 mm i.d. × 30 m) WCOT coated with a 0.20-μm film of SP-2330. Chromatography was performed at 220 °C isothermally. Nitrogen was used as carrier at a flow rate of 1 mL min⁻¹. The split ratio was 80:1. The injector and detector temperature was 240 °C. Sugar assignment was confirmed by gas-liquid chromatography–MS performed on a GCMS-QP 5050A gas chromatograph/mass spectrometer (Shimadzu).
Chromatography was performed on the SP-2330 capillary column run isothermally as indicated above. The He total flow rate was 4.4 mL min⁻¹, the head pressure 12 p.s.i., the injector temperature 250°C, and the split ratio 10:1. Mass spectra were recorded over a mass range of 30 to 600 atomic mass units (Daltons), using an ionization potential of 70 eV.

For NMR analysis, carbon-13 heteronuclear multiple quantum coherence determinations were performed using a Bruker AM 500 spectrometer provided with a 5-mm probe at room temperature. The polysaccharide sample (15 mg) was dissolved in Me₂SO-d₆. Spectra were done at 25°C. Chemical shifts are expressed in ppm (δ) relative to resonance of Me₂SO-d₆ at δ 39.70 (13C) and 2.40 (1H). The major peak was pooled, lyophilized, and resuspended in water for further analysis.

For AFM imaging, the EPS was diluted to 1 ng/µL in milliQ water, and 20 µL of the mix was deposited onto freshly cleaved muscovite mica. After 2 to 5 min, the sample was gently washed with 0.5 mL milliQ water to remove molecules that were not firmly attached to the mica and blow dried with nitrogen. Tapping-mode AFM was performed using a NanoScope III Multimode-AFM (Digital Instruments, Veeco Metrology) with a J-type piezoelectric scanner with a maximal lateral range of 120 µm. Microfabricated silicon cantilevers of 125 µm in length and a force constant of 40 Nm⁻¹ were used (NanoDevices, Veeco Metrology). Cantilever oscillation frequency was tuned to the resonance frequency of the cantilever (280 to 350 kHz). After a period of 15 to 30 min of thermal relaxation, initial engagement of the tip was achieved at scan size zero to minimize sample deformation and tip contamination. The images (512 x 512 pixels) were captured with a scan size of between 0.5 and 3 µm at a scan rate of 1 to 2 scan lines per second. Images were processed using Nanoscope software (Digital Instruments).

RT-PCR
Total RNA was extracted from leaves using the RNeasy plant mini kit according to the manufacturer’s recommendations (Qiagen). RNAs were treated with DNase-free RNaseA. First-strand cDNA was synthesized from 2 µg total RNA using Superscript II reverse transcriptase (Invitrogen). Primers described in Supplemental Table 2 online were used to amplify the targets. qPCR was performed using the Eva Green method according to the manufacturer’s recommendations (Bio-Rad). Melting curves were determined using the dissociation curve software SDS 2.2.2 to ensure that only a single product was amplified. The ABI PRISM 7500HT sequence detection system (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 10 min at 95°C followed by 40 cycles alternating between 15 s at 95°C and 1 min at 60°C. All reactions were run in technical triplicate for each biological replicate, and the average values were used for quantification. The relative quantification of target genes was normalized using the ΔΔCt method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control actin (ΔCt = Ct_target − Ct_actin) and compared with a calibrator (ΔΔCt = ΔCt_sample − ΔCt_calibrator). Relative expression (RQ) was calculated using the sequence detection system SDS 2.2.2 software (Applied Biosystems) and the formula RQ = 2−ΔΔCt

RT-PCR was performed to confirm the levels of Sl NPR1 and At NPR1 mRNA in tomato transgenic plants previously published using the specific primers described in Supplemental Table 2 online (Lin et al., 2004).

SA and JA Quantifications
Phenolic compounds were extracted and analyzed using HPLC according to Daayf et al. (1997). Further SA identity confirmation was performed using UPLC-MS/MS (El Hadrami and Daayf, 2009), and quantification was performed using HPLC. JA was extracted and analyzed using UPLC-MS/MS as described previously (Segarra et al., 2006).

Statistical Analysis
Statistical analysis was performed using the GLM procedure of the SAS 9.1 statistical package. Our data were subjected to either one- or two-way analysis of variance followed by a comparison of the means according to a least significant difference test at P < 0.05 unless otherwise stated. Pairwise comparisons with lower numbers of treatments were conducted according to Student’s t test at P < 0.001.

Accession Number
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number AT1G64280 (At NPR1).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Resistance of Tomato to B. cinerea B8403 Isolate Requires Jasmonic Acid Signaling.

Supplemental Figure 2. EPS Production by Different Isolates of B. cinerea and Disease Development on Tomato Leaves.

Supplemental Figure 3. Characterization of Susceptibility Induced by B. cinerea EPS in Tomato.

Supplemental Figure 4. EPS-Induced Tomato Susceptibility Does Not Occur in JA Mutants def1 and spr2.

Supplemental Figure 5. B. cinerea EPS Confers Resistance to P. syringae DC 3000 in Tomato.

Supplemental Table 1. B. cinerea Isolates Used in This Study.

Supplemental Table 2. Primers Used in This Study.

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