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**Note**

# Identification of *Arabidopsis* accession with resistance to *Botrytis cinerea* by natural variation analysis, and characterization of the resistance response

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**Abstract** *Botrytis cinerea* is a ubiquitous necrotrophic fungal pathogen that infects over 200 different plant species. We have analyzed 17 *Arabidopsis* ecotypes for natural variations in their susceptibility to *B. cinerea*, and found compatible and incompatible *Arabidopsis–Botrytis* interactions. We determined that *Arabidopsis* ecotype L*er* is resistant to 5 *B. cinerea* isolates used in this study. To further investigate the roles of the salicylic acid (SA)-dependent defense response pathways against *B. cinerea*, we inoculated various *Arabidopsis* mutants with the pathogen. *Arabidopsis* L*er* plants expressing the *nahG* gene inoculated with *B. cinerea* showed as much resistance as the parental plants (L*er*-wild type). The *sgt1b-1* and *rar1-10* mutants also showed resistance to the pathogen. In this study, we discuss the natural variations in the symptoms observed among various ecotypes upon inoculation with *B. cinerea*. In addition, SA plays only a minor role in preventing systemic infection with *B. cinerea*.

**Key words:** *Arabidopsis*, *Botrytis cinerea*, gray mold, natural variation, salicylic acid.

Plants interact with various types of microbes, only a few of which actually harm them. Plant diseases rarely occur because plants have evolved sophisticated defense mechanisms against potential pathogens. Many plants defend themselves against microbial pathogens by activating both localized and systemic resistance responses. The recognition of the invading pathogen by the plant at an early stage of infection is crucial. Specific recognition is thought to be mediated through direct or indirect interactions between the product of a resistance (*R*) gene in the plant and product of a corresponding avirulence (*avr*) gene in the pathogen.

Three major signaling pathways have been identified, mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Hammond-Kosack and Jones 1996; Ryals et al. 1996). These signaling pathways result in induction of various defense responses aimed at restricting pathogen growth and symptoms, including accumulation of antimicrobial compounds/proteins and expression of defense-related genes. *Arabidopsis thaliana* has been extensively used as a model organism for plant pathology studies, and can be colonized by both biotrophic and necrotrophic pathogens (Glazebrook et al. 1997; Thomma et al. 1998). SA-dependent signaling is found to activate the defense response pathways that primarily confer resistance to bacterial or biotrophic fungal pathogens. In contrast, ET and JA have been implicated in defense responses to necrotrophic pathogens in a few plant species (Ferarri et al. 2003; Thomma et al. 1999a). Necrotrophs obtain nutrients from dead or dying cells. *Botrytis cinerea*, the causal agent of gray mold, is a ubiquitous necrotrophic fungal pathogen that infects over 200 different plant species (Elad 1997). Infection of *Arabidopsis* plants with *B. cinerea* induces a subset of defense genes that are not induced by SA, including the *PDF1.2* gene, which encodes defensin, an antifungal protein (Penninckx et al. 1998; Thomma et al. 1998; 1999a). Induction of *PDF1.2* is blocked in *ein2* and *coi1* mutants (Penninckx et al. 1996; Zimmerli et al. 2001), which are defective in ET- or JA-signal transduction pathways, respectively (Feys et al. 1994; Guzman and Ecker 1990). JA insensitivity conferred by the *coi1* mutant and ET insensitivity conferred by the *ein2* mutant depresses the resistance of the respective plants to *B. cinerea* infection (Thomma et al. 1998; 1999a). Consistent with these data, *B. cinerea* infection fails to induce SA

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accumulation or SAR (systemic acquired resistance) in *Arabidopsis* (Govrin and Levine 2002). However, several reports show that SA or its analog BTH (benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester) can induce resistance to *B. cinerea* in several plant species, including bean, tobacco, and tomato (Audenaert et al. 2002; De Meyer et al. 1999; Murphy et al. 2000). In addition, *Arabidopsis* local resistance to *B. cinerea* involves SA and phytoalexin, and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5*, or *PAD4* (Ferrari et al. 2003).

To understand the genetic and molecular basis of plant-pathogen interactions, we utilize natural variations of *Arabidopsis* to study the genetics of resistance to *B. cinerea*. *A. thaliana* provides a genetically amenable system in which to examine the various components of disease resistance (Koch and Slusarenko 1990). Several accessions (ecotypes) of *A. thaliana* are available, which differ genetically because of selection pressures imposed on them by their different environments of origin (Kagan and Hammerschmidt 2002). These differences include variation in resistance to pathogens. For example, ecotypes of *Arabidopsis* differ in their ability to resist infection by the club root pathogen *Plasmodiophora brassicae* (Fuchs and Sacristan 1996), cauliflower mosaic virus (Callaway et al. 1996; Leisner and Howell 1992), turnip crinkle virus (Dempsey et al. 1997), bacterium *Xanthomonas campestris* pv. *campestris* (Tsuji et al. 1991), oomycetes *Albugo candida* (Holub et al. 1995), *Peronospora parasitica* (Mauch-Mani et al. 1993), and hemibiotrophic fungal pathogen *Colletotrichum higginsianum* (Narusaka et al. 2004; 2009, O'Connell et al. 2004). Resistance to these pathogens is because of the presence of 1 or more *R* genes. The types of *R* genes varied among ecotypes, which may be a reflection of variation in disease pressure in the locations where the ecotypes were collected (Kagan and Hammerschmidt 2002). Therefore, to determine the components of the host response to necrotrophic infection, mutants with enhanced susceptibility to *B. cinerea* were identified. Veronese et al. (2004) reported that the *BOS* loci in *Arabidopsis* were required for resistance to *B. cinerea* infection. Some of their loci may affect camelaxin levels and responsiveness to ET and JA. In this study, we describe variation in the symptoms observed among various ecotypes following inoculation with *B. cinerea*. We also show SA plays a minor role in preventing systemic infection with *B. cinerea*.

Isolates of *B. cinerea* Persoon isolated from *Brassica campestris* L. (MAFF237695) and *Lactuca sativa* L. (MAFF305538) were provided by NIAS Genebank. An isolate of *B. cinerea* obtained from *Cucumis sativus* L. (kumiai-chem BC1) was provided by Kumiai Chemical Industry Co., LTD. Isolates of *B. cinerea* obtained from *Solanum lycopersicum* L. (Ibaen-04016 and Ibaen-04042) were provided by Horticultural Institute, Ibaraki Agricultural Center. Cultures of the isolate were maintained on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 24°C in the dark. To obtain spores, *B. cinerea* mycelia were placed on PDA and incubated at 24°C in the dark for 3–4 days, and then incubated at 24°C under continuous black light form blue lamps (FL10BLB; Toshiba Corp., Tokyo, Japan) for 2–3 days. Conidia were then obtained by gentle scraping of cultures. The spore suspension was filtered through two layers of sterile cheesecloth and, the spores were counted on a hemacytometer slide.

*A. thaliana* ecotype Columbia (Col-0) plants were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. For the intact plant assay, whole plants were inoculated with five different strains of *B. cinerea* (MAFF237695, MAFF305538, kumiai-chem BC1, Ibaen-04016, and Ibaen-04042) spore suspension ($1.5\times10^5$ spores $ml^{-1}$) in 1/2 potato dextrose broth (PDB) (Difco, Detroit, MI, USA). Inoculated plants were then placed in a growth chamber at 22°C with a 12-h light/12-h dark cycle and maintained at 100% relative humidity. Control plants were treated only with 1/2 PDB or distilled water. When challenged with their fungi, Col-0 plants developed brown necrotic lesions. These lesions had expanded from the inoculation site by 2 days post inoculation (dpi), and subsequently spread over the entire leaf (Figures 1, 2). The fungus sporulated on the host and recovered from the infected plant material was used to complete Koch's postulates. There were no obvious differences among the fungal strains with respect to the symptoms they induced; therefore, only one among them (MAFF237695) was chosen for further experiments.

*Arabidopsis* ecotypes, with Gr-1 and St-0, were obtained from SENDAI *Arabidopsis* Seed Stock Center (SASSC). Seeds from Aa-0, AUA/RHON, Bensheim, DijonG, Ei-2, Greenville, Hi-0, Kendalville, Mühlen, Niederzenz, and S96 were purchased from Lehle Seeds (TX, USA). The Col-0, L*er*, Nos, and RLD were obtained from RIKEN BRC. To investigate natural variations in *B. cinerea* susceptibility, we tested 17 *Arabidopsis* ecotypes mentioned above for susceptibility to *B. cinerea* (MAFF237695). We evaluated the disease reactions of *Arabidopsis* ecotypes to *B. cinerea* based on certain aspects, such as the extent of pathogen colonization of the host as evaluated by lactophenol-trypan blue staining, and the degree of host necrosis. In addition, the lesion size has been used as a parameter in several studies to indicate the plant's susceptibility to *B. cinerea* (Ferarri et al. 2003; Govrin and Levine 2000; Mengiste et al. 2003; Denby et al. 2004). One or two 5-$\mu$l drops of the spore suspension ($1.5\times10^5$ spores $ml^{-1}$ in 1/2 PDB) were placed on each attached leaf of *Arabidopsis* plants without wounding. The lesion diameter varied considerably among the *Arabidopsis* ecotypes with the

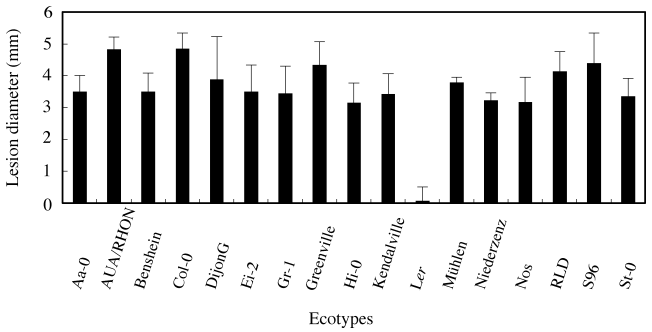


Figure 1. Lesion diameters on leaves of various *Arabidopsis* ecotypes, 2 days after inoculation with *B. cinerea*. Attached leaves of 28- to 30-day-old plants were inoculated by placing one or two 5-$\mu$l drops of a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) on each leaf without wounding. The data are obtained from more than 10 plants of each ecotype. The experiment was repeated at least twice.

2-day lesion size ranging from 0 mm for L*er* to 8 mm for Col-0 (Figure 1). Control plants inoculated with 1/2 PDB instead of the fungus did not develop any lesions. Most of the interactions observed with these ecotypes were compatible. However, an incompatible phenotype was found following inoculation of ecotype L*er*, which appeared to be resistant, developing only small necrotic flecks at the inoculation sites by 3 dpi that did not expand further (Figure 2). Spore germination was observed microscopically on the surface of L*er* plants, and none of the sporelings entered the plant cells. No cell death appeared on inoculated leaves 2 dpi (Figure 2). The L*er* plants were inoculated with five different isolates of *B. cinerea* (MAFF237695, MAFF305538, kumiai-chem BC1, Ibaen-04016 and Ibaen-04042). L*er* plants developed almost no symptoms, even after 5 days of inoculation (data not shown).

$F_2$ progeny from a test cross between L*er* and Col-0 segregated 63 : 548 for resistance versus susceptibility to *B. cinerea* (MAFF237695). Thus, L*er* does not appear to have a single dominant allele that confers resistance to *B. cinerea*. On the contrary, Denby et al. (2004) showed that all 16 *Arabidopsis* ecotypes, containing L*er*, were susceptible to *B. cinerea*, and several QTL responsible for *B. cinerea* susceptibility were identified. As Denby et al. suggested that different mechanisms govern defense against two *Botrytis* isolates from grape and pepper, defense against five isolates used here may be different from isolates used by Denby et al.

Attempts by microbial pathogens to infect plants trigger the activation of a defense signaling network. *Arabidopsis* has three main defense-related pathways, namely SA-, JA-, and ET-mediated pathways. In this study, we investigated the effect of ethephon, and BTH application on the induction of resistance to *B. cinerea*. The Col-0 plants were pre-treated with 1 mM ethephon, or 0.5 mM BTH, and then were inoculated with *B. cinerea* 24 h later. The ethephon-treatment protected Col-0 plants against *B. cinerea* when observed 3 days after inoculation (Figure 3); however, BTH-treatment caused severe disease symptoms as compared with the control.

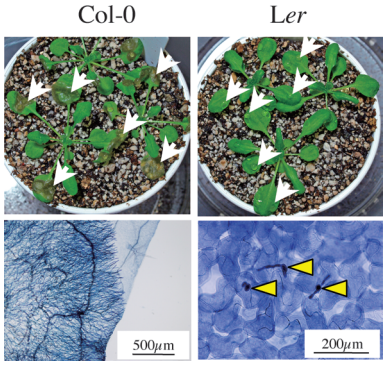


Figure 2. Infection phenotypes of *Arabidopsis* leaves inoculated with the fungus *B. cinerea*. Attached leaves of 28- to 30-day-old plants were inoculated by placing two 5-$\mu$l drops of a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) on each leaf without wounding. Lesion phenotypes at 2 dpi are shown. Leaves are harvested at 2 dpi, and stained with trypan blue. Arrows indicate the inoculation sites and lesions. Arrow heads indicate the germed spores. Each picture shows a representative of three independent experiments.

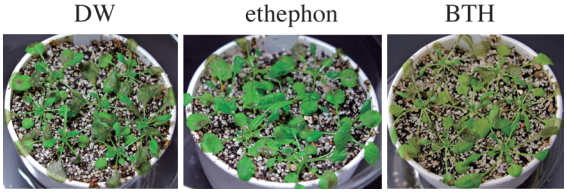


Figure 3. The effect of direct applications of ethephon, and BTH on induction of resistance to *B. cinerea*. The Col-0 plants were pre-treated with 1 mM ethephon, or 0.5 mM BTH, and were sprayed with a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) 24 h later. Lesion phenotypes at 3 dpi are shown. Each picture shows a representative of three independent experiments.

The results indicated that ethephon protected plants against *B. cinerea* attack. However, the efficacy of BTH is limited. BTH-treatment may suppress JA/ET-dependent defenses because the SA- and JA- pathways appear to be antagonistic (Rao et al. 2000; Seo et al. 1997; Shah et al. 1999) while the JA- and ET-pathways generally act synergistically (Penninckx et al. 1998). Our results agree

with those reported by Thomma et al. (1999a). Govrin and Levine (2002) also demonstrated that the treatments with SA and BTH failed to inhibit *B. cinerea* growth. Several reports suggest that SA-signaling also plays a role in resistance to *B. cinerea* (Ferrari et al. 2003; Govrin and Levine 2002).

We investigated induction of the pathogen-inducible genes *PR-1* (Uknes et al. 1992); *PR-4*, which encodes a hevein-like protein (Potter et al. 1993); and *PDF1.2* (Penninckx et al. 1996), in L*er* and Col-0 plants by using qRT-PCR analyses. *PR-1* is an indicator for SA-dependent defense responses (Delaney et al. 1994; Penninckx et al. 1996; Thomma et al. 1999b), while *PR-4* and *PDF1.2* are indicators for JA/ET-dependent responses (Penninckx et al. 1996; 1998; Thomma et al. 1998; 1999b).

Total RNA was isolated and treated with RNase-free DNase (Promega, WI, USA). 500 ng of total RNA was synthesized with oligo dT primer using a PrimeScript RT reagent kit (Takara, Otsu, Japan). qRT-PCR was performed with SYBR Green PCR Master Mix (BIO-Rad Laboratories, CA, USA) using the first-strand cDNA as a template on an MJ Opticon (Bio-Rad Laboratories). qRT-PCR mixtures consisted of 1xSYBR Green I PCR Master Mix and 200 nM (each) sense and antisense primers. Following a preliminary denaturation step at 95°C for 30 s, the reaction mixtures were cycled 40X at 95°C for 5 s and at 65°C for 20 s. The target sample copy number was averaged for two reactions, and the experiment was repeated twice. The expression of *CBP20* gene was used for normalization as a standard control gene. Nucleotide sequences of gene-specific primers were as follows: *CBP20* (At5g44200; forward primer 5′-CCT TGT GGC TTT TGT TTC GTC -3′, reverse primer 5′-TGT TTC GTC CTG TTC TAC TC-3′); *PR-1* (At2g14610; forward primer 5′-CCC ACA AGA TTA TCT AAG GGT TCA C-3′, reverse primer 5′-CCC TCT CGT CCC ACT GCA T-3′) (Jirage et al. 2001); *PR-4* (At3g04720; forward primer 5′-CCT TGT TGA TAG CCA AAA CCA TC-3′, reverse primer 5′-TTG GTA GTC AAC AAT GAG ATG -3′); *PDF1.2* (At5g44420; forward primer 5′-TGT CCC ACT TGG CTT CTC G-3′, reverse primer 5′-CCA TCA TCA CCC TTA TCT TCG C-3′). The gene expression is shown as relative values set at a value of 1 in the control plants. This experiment was repeated twice with similar results.

Transcript levels of *PR-1* increased with time in the L*er* but slight induction by 24 hpi in the Col-0 plant (Figure 4). Expression of the *PR-4* gene in L*er* plants inoculated with *B. cinerea* increased between 0 and 24 hpi. In the inoculated Col-0 plants, the expression of *PR-4* also increased with time, but induction of *PR-4* was weaker and slower than that of L*er*. Transcript levels of *PDF1.2* also increased between 0 and 24 hpi in both the L*er* and Col-0 plants, but induction of *PDF1.2*

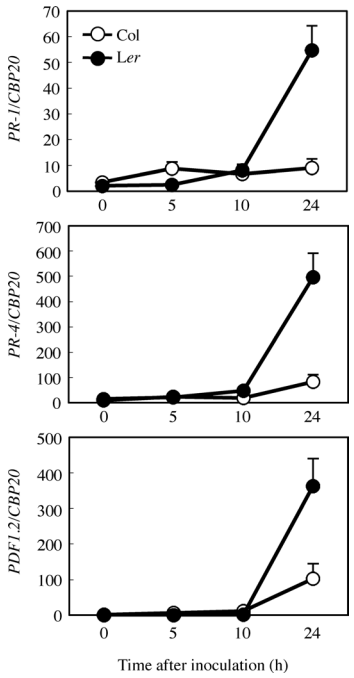


Figure 4. Quantitative real-time PCR analysis of expression of *PR-1*, *PR-4*, and *PDF1.2* genes. The 28- to 30-day-old *Arabidopsis* plants were sprayed with a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) and harvested 5, 10, or 24 h later. As a control, leaf material was also collected just before inoculation (0). The experiment was repeated at least twice.

in the Col-0 was weaker than that of L*er*. The defense related genes were expressed during development of *B. cinerea* spores deposited on L*er* leaf surfaces, contained spore germination and restricted hyphal growth but not penetration during the first 24 h after inoculation.

To further investigate the roles of the SA-dependent defense response pathways against *B. cinerea,* we inoculated the pathogen into various *Arabidopsis* mutants (Figure 5). Mutant lines defense no death (*dnd1*) (Nos background), that was obtained from a collection of *Ds* transposon-tagged lines (Kuromori et al. 2004), and *cpr5-2* (approximately 87% Col-0 and 13% Nos in chromosomal composition) plants exhibit high levels of SA (Bowling et al. 1997; Yu et al. 1998), while L*er-nahG* (L*er* background) fails to accumulate SA (Gaffney et al. 1993). Susceptibility to *B. cinerea* was determined using an entire plant assay. One or two 5-$\mu$l drops of the spore suspension ($1.5\times 10^5$ spores ml$^{-1}$ in 1/2 PDB) were placed on each attached leaf without wounding. The lesion diameter varied considerably among mutants with the 2-day lesion size ranging from 0 mm for L*er-nahG* to 5 mm for *cpr5-2*. Control plants inoculated with 1/2 PDB instead of the fungus did not develop any lesions, while the *dnd1* and *cpr5-2* mutants inoculated with *B. cinerea* developed necrotic lesions 2 days after inoculation. These lesions had expanded from the inoculation site by 2 dpi, and subsequently spread over the entire leaf. In contrast, L*er-nahG* plants and wild-type L*er* plants did not develop

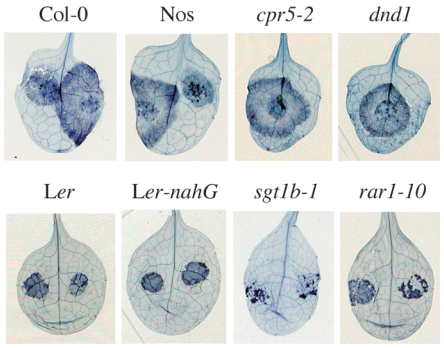


Figure 5. Infection phenotypes of *Arabidopsis* mutants inoculated with the fungus *B. cinerea*. Attached leaves of 28- to 30-day-old plants were inoculated by placing one or two 5-$\mu$l drops of a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) on each leaf without wounding. Leaves are harvested at 2 dpi, and stained with trypan blue. Each picture shows a representative of three independent experiments.

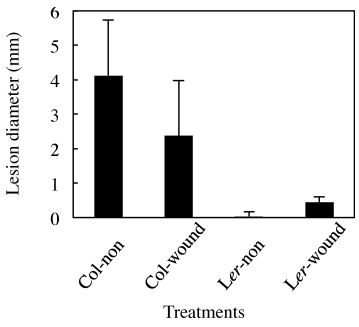


Figure 6. Lesion diameters on leaves of different *Arabidopsis* ecotypes 3 days after inoculation with *B. cinerea*. Attached leaves of 28- to 30-day-old plants were inoculated by placing one to two 5-$\mu$l drops of a spore suspension of *B. cinerea* strain MAFF237695 ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) on each leaf with or without puncture wound using a 25G needle. The data are obtained from more than 10 plants. The experiment was repeated at least twice. Col-non and L*er*-non, inoculation without wounding; Col-wound and L*er*-wound, inoculation with wounding.

necrotic lesions. The *sgt1b-1* and *rar1-10* mutants (L*er* background) also developed no lesion when inoculated with one 5-$\mu$l drop of the spore suspension by 2 dpi.

In this study, *Arabidopsis* L*er* plants expressing the *nahG* gene inoculated with *B. cinerea* showed as much resistance as the parental plants (L*er*-wild type). It has been reported that the *nahG* plants did not show increased susceptibility compared to wild-type plants (AbuQamar et al. 2006; Veronese et al. 2004). On the contrary, it has been reported that *nahG* plants had enhanced lesion formation at the site of *B. cinerea* infection (Ferarri et al. 2003; Govrin and Levine 2002). We demonstrated that the *dnd1* and *cpr5-2* mutants inoculated with *B. cinerea* began to develop necrotic lesions 2–3 days after inoculation. Other groups also reported that *ssi2* mutants, that lose a stearoyl-ACP desaturase activity accompanied by the constitutive accumulation of elevated SA level, confer susceptibility to *B. cinerea* (Kachroo et al. 2001; Nandi et al. 2005). These results indicate that SA-signaling plays only a minor role in resistance against *B. cinerea* in *Arabidopsis*.

Govrin and Levine (2000) proposed that cell death induced by *B. cinerea* is an important component of virulence, since *Botrytis* promotes and benefits from host cell death. The expression of the 2 plant signaling components EDS1 and SGT1, which are required for HR-dependent resistance, enhanced the resistance to *B. cinerea* in *Nicotiana benthamiana* (Oirdi and Bouarab 2007). However, we showed that the *dnd1* mutant, which does not show HR cell death, developed lesions by inoculation with the necrotrophic fungal pathogen *B. cinerea*. We also showed that the *sgt1b-1* and *rar1-10* mutants (L*er* background) did not develop necrotic lesions by inoculation with *B. cinerea*. The SGT1 and RAR1 are important signaling components of *R* gene-mediated disease resistance (Azevedo et al. 2002). The present data indicate that defense responses against *B. cinerea* in L*er* can be activated via *R* gene-independent defense pathway.

There are some discrepancies between our results and that of other studies (Denby et al. 2004; Govrin and Levine 2002). A possible explanation for this could be the use of a different pathogen strain, especially considering that different *B. cinerea* strains can exhibit variable degree of aggressiveness even on the same host (Govrin and Levine 2002). It seems that the differences may also have arisen because of the method adopted for inoculation with *B. cinerea*. In our study, the inoculum was placed on each attached leaf without wounding, while in the previous study the inoculum was placed on each attached leaf punctured with a needle (Govrin and Levine 2000; 2002) or on each detached leaf without wounding (Denby et al. 2004). Liu et al. (2007) reported that defense systems are less responsive in detached leaves than in intact plants. The loss of a systemic defense response in detached leaves likely is associated with increased susceptibility. On the other hand, wounding is often used to inoculate necrotrophic pathogens into leaves of host plants. It is supposed that wounding facilitates infection by necrotrophic pathogens. Therefore, we performed inoculation with *B. cinerea* by wounding and non-wounding methods (Figure 6). One or two 5-$\mu$l drops of the spore suspension ($1.5\times10^5$ spores ml$^{-1}$ in 1/2 PDB) were placed on each attached leaf with wounding (Figure 6). A single puncture performed with a syringe needle induced moderate resistance against *B. cinerea*. Although the lesion sizes of Col-0 with the wounding method were slightly smaller than those for the non-wounding method, its compatibility to *B. cinerea* is not controlled by inoculation methods. In addition, lesion sizes of L*er* with the wounding method were only wound flecks. Recently, Chassot et al. (2008) reported that hyphal

growth of *B. cinerea* in wounding leaves was strongly inhibited compared to unwounded control. Because wounding of leaf surface provides entrance for invading pathogen, including necrotrophic and bacteria, plants respond to the injury by localized defense responses (Reymond et al. 2000). Therefore, wounding inoculation may cause activation of plant defense mechanisms because of the wounding stress.

In summary, *B. cinerea* induced a defense response in *Arabidopsis*, mediated by ET-signaling pathway. To understand the genetic and molecular basis of plant-pathogen interactions, ecotypes of *Arabidopsis* that differ in their ability to resist infection by *B. cinerea* are very helpful. We also showed that SA plays only a minor role in preventing systemic infection with *B. cinerea*. The gray mold on the model plant *Arabidopsis*, caused by *B. cinerea* infection, provides a valuable new genetic system for analysis of fungal pathogenicity factors as well as of host responses in a necrotrophic disease interaction.

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