Detached and Attached *Arabidopsis* Leaf Assays Reveal Distinctive Defense Responses Against Hemibiotrophic *Colletotrichum* spp.

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Submitted 8 May 2007. Accepted 19 June 2007.

The agriculturally important genus *Colletotrichum* is an emerging model pathogen for studying defense in *Arabidopsis*. During the process of screening for novel pathogenic *Colletotrichum* isolates on *Arabidopsis*, we found significant differences in defense responses between detached and attached leaf assays. A near-adapted isolate *Colletotrichum linicola* A1 could launch a typical infection only on detached, but not attached, *Arabidopsis* leaves. Remarkably, resistance gene-like locus *RCH1*-mediated resistance in intact plants also was compromised in detached leaves during the attacks with the virulent reference isolate *C. higginsianum*. The differences in symptom development between the detached leaf and intact plant assays were further confirmed on defense-defective mutants following inoculation with *C. higginsianum*, where the greatest inconsistency occurred on ethylene-insensitive mutants. In intact *Arabidopsis* plants, both the salicylic acid- and ethylene-dependent pathways were required for resistance to *C. higginsianum* and were associated with induced expression of pathogenesis-related genes *PR1* and *PDF1.2*. In contrast, disease symptom development in detached leaves appeared to be uncoupled from these defense pathways and more closely associated with senescence: an observation substantiated by coordinated gene expression analysis and disease symptom development, and chemically and genetically mimicking senescence.

Additional keywords: anthracnose, biotrophy, necrotrophy, nonhost resistance.

The large Ascomycete genus *Colletotrichum* is one of the most economically important groups of plant pathogens, causing anthracnose disease or blight worldwide (Bailey and Jeger 1992). The *Arabidopsis*–*Colletotrichum* spp. pathosystem is an attractive model system for the study of fungal pathogen–plant interactions (Narusaka et al. 2004; O’Connell et al. 2004; Perfect et al. 1999; Shimada et al. 2006). This pathosystem benefits not only from tremendous genetic resources of the

host (Alonso et al. 2003) but also from the pathogen’s unique advantages: it is a haploid organism for most of its life cycle, it can be cultured axenically, and it can be manipulated genetically (transformation and gene disruption) (Epstein et al. 1998; Redman et al. 1999; Thon et al. 2000). More importantly, many *Colletotrichum* spp. use a hemibiotrophic infection strategy, in which the pathogen initially grows as a biotroph in living host cells before switching to a destructive necrotrophic mode of infection (Perfect et al. 1999; Wharton et al. 2001). Therefore, *Colletotrichum* spp. possess features of obligate biotrophic pathogens, which require a living host tissue during parasitism, and features of necrotrophic pathogens, which derive energy from killed cells. Thus, the genus *Colletotrichum* is attractive as a model fungus for studying infection structures, fungal lifestyles, and determinants of the switch from biotrophy to necrotrophy (Latunde-Dada 2001; Perfect and Green 2001).

Genetic analyses of defense responses in the model plant *Arabidopsis thaliana* have revealed potential links between the lifestyle of the pathogen and defense signaling pathways, based on whether the defense against that pathogen is controlled via the salicylic acid (SA) or jasmonate/ethylene (JA/ET) pathways (Glazebrook 2005; Oliver and Ipcho 2004). It has been suggested that effective defense against biotrophic pathogens is due largely to the hypersensitive response in the host, and to the associated activation of defense responses regulated by SA-dependent pathways (Glazebrook 2005). In contrast, defense against necrotrophic pathogens is mediated mainly through JA/ET-dependent signaling. Increasing evidence has shown that SA- and JA/ET-dependent signaling pathways appear to interact in a complex fashion, with the primary mode of interaction being mutual antagonism (Feys and Parker 2000; Kunkel and Brooks 2002; Penninckx et al. 1998). Microarray analyses have shown that a considerable number of genes are coregulated by SA and JA/ET (Schenk et al. 2000). Interpathway communication (crosstalk) has been speculated to help plants fine tune and prioritize defense responses upon encountering multiple signals (Feys and Parker 2000; Kunkel and Brooks 2002). Compared with biotrophic and necrotrophic pathogens, the knowledge of defense pathways for hemibiotrophs such as the genus *Colletotrichum* is limited and, therefore, characterization of the defense responses against this group would aid in its classification as a biotroph, a necrotroph, or a separate group altogether (Oliver and Ipcho 2004).

Most *Colletotrichum* spp. possess two distinct nutritional phases during infection because they must undergo a metabolic switch from biotrophic to necrotrophic growth. In the

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*The e-Xtra logo stands for “electronic extra” and indicates additional information is available online. Figures 2 through 7 appear in color and two supplementary tables are published online.

screening for genes that might play such roles, a GAL4-like transcriptional activator gene *Colletotrichum lindemuthianum* transcriptional activator 1 (*CLTA1*) was discovered and shown to be both a pathogenicity gene and a regulatory gene involved in the biotrophy-to-necrotrophy switch process (Dufresne et al. 2000). Our previous study, using a glycerol-3-phosphate dehydrogenase disrupted strain (Δ *gpdh*), provided evidence that glycerol, rather than other common energy forms such as sugar and amino acids, was a major nutrient sustaining intracellular hyphal growth of *C. gloeosporioides* (Wei et al. 2004). A resistance (*R*)-gene-like locus has been identified in the *Arabidopsis* ecotype Eil-0 to *C. higginsianum* (Narusaka et al. 2004). Using adapted and nonadapted *Colletotrichum* spp. and a fungal mutant with impaired penetration ability, Shimada and associates (2006) demonstrated that nonhost resistance to nonadapted species was accomplished through the accumulation of papillary callose. They also showed that actin filament function, but not the PEN1 syntaxin, a homolog of barley ROR2 indispensable for papilla resistance to biotrophic fungus *Blumeria graminis* f. sp. *hordei* (Collins et al. 2003), is essential for this preinvasion resistance at the cell periphery.

To develop new pathosystems for the analysis of hemibiotrophy and to dissect defense mechanisms during hemibiotrophic infection, we screened a collection of *Colletotrichum* spp. for pathogenicity analysis on *Arabidopsis*. We found a significant variation in the interaction phenotype between both detached-leaf and intact-plant assays, and among defense-impaired mutants upon inoculation with the pathogens. These results stress the potential effects of methodology on the analysis of pathogenicity and defense responses and add a layer of complexity and caution for interpretation if using detached leaves only.

RESULTS

Nearly adapted *C. linicola* A1 causes typical hemibiotrophic infection on detached *Arabidopsis* leaves but fails to penetrate attached leaves.

To identify novel *Colletotrichum* spp. capable of infecting *Arabidopsis*, we selected 20 *Colletotrichum* isolates collected from different host species for pathogenicity on detached *Arabidopsis* Columbia (Col-0) leaves. Only one isolate, 93-121 A1, from field bindweed (*Convolvulus arvensis*), caused

macroscopic disease symptoms. To identify this isolate, we compared its ribosomal (r)DNA internal transcribed spacer (ITS) regions (ITS1 and ITS2) with those of other known species. This region has been widely used for classification of organisms, including pathogenic *Colletotrichum* spp. (Moriwaki et al. 2002; Sreenivasaprasad et al. 1996). The ITS region of isolate 93-121 A1 was identical to two reported *Colletotrichum linicola* strains (AB046609 and Z32984) and clustered with more than 99% identity to *C. higginsianum* (AB042302 and AB042303) and *C. destructivum* (AJ558109, AJ558110, and AJ558111); the latter two species were reported to be virulent in *Arabidopsis* (Narusaka et al. 2004; O'Connell et al. 2004) (Fig. 1; Supplementary Table S1). After comparing the morphological characteristics of fungal cultures with reference species (data not shown) and rDNA sequencing, we hereafter referred to this isolate as *C. linicola* A1.

To further characterize *C. linicola* A1 pathogenicity on *Arabidopsis*, a detailed comparison was conducted against the virulent strain *C. higginsianum* Sacc. (IMI349061) originating from *Brassica rapa* (CABI Bioscience, Egham, Surrey, U.K.). *C. linicola* A1 caused typical disease symptoms (necrotic lesions surrounded by chlorotic halos) on detached leaves of *Arabidopsis* ecotype Col-0, and was notably more aggressive in older leaves than in younger leaves (Fig. 2A). No macroscopic symptoms were visible until 2 days postinoculation (dpi). The inoculation sites developed a water-soaked appearance by 6 dpi (Fig. 2A). To monitor the process of fungal infection, leaf samples were stained with trypan blue at intervals postinoculation and examined microscopically (Fig. 2B). Unicellular conidia became septate during germination and produced melanized, globular appressoria at 1 dpi, which penetrated the epidermal cells and formed infection vesicles (iv) at approximately 2 dpi. By 3 dpi, the infection vesicles had developed large primary hyphae (ph) (Fig. 2B). Slim secondary hyphae (sh) emerging from the larger primary hyphae could be observed at 4 dpi, which coincided with the onset of disease symptoms, indicating a switch from biotrophy to necrotrophy. Interestingly, the development of some infection structures (iv, ph, and sh) in *C. linicola* A1 appeared to be faster than those of *C. higginsianum*, although there were no obvious differences in the speed of symptom development (Fig. 2A and B). At later stages of infection, abundant acervuli (conidia-forming structures) were produced on the surface of infected host tissues,

Fig. 1. Phylogenetic relationships between the *Colletotrichum* isolates used for pathogenicity test in *Arabidopsis* and other representative species. The unrooted tree was constructed using the ClustalW package online based on the sequences of internal transcribed spacer (ITS1) region of ribosomal DNA (inset graph). Scale bar is a distance corresponding to approximately two base changes per 100 nucleotide positions. The two isolates *Colletotrichum linicola* A1 and *C. higginsianum* are highlighted (bold). Arrows indicate the position of primers used for amplification of ITS1 and ITS2. The schematic positions of 5.8S, 18S, and 28S rDNA (solid blocks) also are indicated.

indicating a completion of the asexual lifecycle (Fig. 2C). To further verify the biotrophic characteristic of primary hyphae of *C. linicola* A1, inoculated tissues were plasmolyzed in 0.8 M NaNO₃ 24 h postinoculation (Fig. 2D). Over a 60-min time course, the cytoplasm and tonoplast of plant cells gradually concentrated, separating from the cell wall, but not binding with the primary hyphae. Collectively, these results demonstrate that the *C. linicola* A1, like *C. higginsianum*, possesses

both biotrophic and necrotrophic phases of its lifecycle, and causes typical infection in detached *Arabidopsis* leaves.

To determine the pathogenicity of *C. linicola* A1 on intact *Arabidopsis* plants, 4-week-old plants were inoculated by either spray or droplet of conidial suspension (Narusaka et al. 2004; O'Connell et al. 2004; Shimada et al. 2006). Surprisingly, no macroscopic disease symptoms were observed following inoculation with *C. linicola* (Fig. 3A), whereas symptoms devel-

Fig. 2. Pathogenicity of *Colletotrichum linicola* A1 and *C. higginsianum* on detached leaves of *Arabidopsis*. **A**, Symptom time course. Five leaves detached from the same plant, aligned by ages (from young to old: 1 to 5), were inoculated with droplets of conidial suspensions of *C. linicola* A1 (C.I.A1), *C. higginsianum* (C.h.), or water (Mock). The inoculated leaves were photographed at 2, 4, and 6 days postinoculation (dpi), respectively. **B**, Cytological examination of fungal development in infected tissues. The inoculated leaves were stained by trypan blue and observed under microscope at 2, 3, and 4 dpi, respectively; ap, appressorium; iv, infection vesicle; ph, primary hyphae; sh, secondary hyphae. Arrowheads indicate the transition site from ph to sh. Scale bar = 10 μm. **C**, Acervulus production of *C. linicola* A1 at later stage of infection on detached leaves. The diseased tissue with *C. linicola* A1 infection was collected and examined using scanning electron microscopy. Scale bar = 20 μm. **D**, Biotrophic hyphae at early infection stages. Leaves 4 dpi with *C. linicola* A1 were treated with 0.8 M NaNO₃ and photographed at 30 min after treatment to monitor the cellular plasmolysis and localization of primary hyphae by focusing on the surface of epidermal cell (left panel) and on the inner body of epidermal cell (right panel), respectively. The insets show the enlarged part of penetration sites. Arrowheads indicate the plasmolyzed cytoplasm; c, conidium. Scale bar = 10 μm.

oped normally in plants inoculated with *C. higginsianum* 3 dpi. These lesions eventually coalesced, resulting in the death of the leaves. None of the *C. linicola A1* inoculations produced disease symptoms on plants at different physiological stages of any *Arabidopsis* wild type or defense-impaired mutants tested. Occasionally, though, marginal hypersensitive response-like dark brown flecks were observed on the leaves (data not shown). Microscopic examinations revealed that *C. linicola A1* appressorium formation appeared normal on wild-type plants (Fig. 3B) as well as on defense-impaired mutants, but infection hyphae generally were not observed, suggesting that a nonhost or basal-like penetration resistance exists in intact leaves and plays a crucial role in the *Arabidopsis* response to *C. linicola A1*. We concluded that the *C. linicola A1* strain lies somewhere between an adapted and nonadapted *Colletotrichum* sp. (Shimada et al., 2006) and the pathogenicity of the strain on *Arabidopsis* plants is conditional: whereas the *C. linicola A1* strain is fully virulent in the detached leaves, active penetration resistance in intact leaves knocks down the fungal pathogenicity, although the underlying cytological and molecular mechanisms behind the phenomena remain to be elucidated (Koh and Somerville 2006; Lipka and Panstruga 2005).

***RCH1*-mediated disease resistance is compromised in detached leaves.**

The striking differences in pathogenicity of *C. linicola A1* on detached and attached leaves prompted us to further investigate whether this was a general phenomenon in *Colletotrichum* spp.–*Arabidopsis* interactions. To determine whether leaf detachment affects *R* gene resistance, we compared disease development on both attached and detached Eil-0 leaves using a virulent reference strain *C. higginsianum*. In the ecotype Eil-0, resistance against *C. higginsianum* is determined by a dominant allele at a locus *RCH1* (Narusaka et al. 2004). After *C. higginsianum* attack, intact Eil-0 plants showed a typical hy-

Fig. 3. Pathogenicity of *Colletotrichum linicola A1* and *C. higginsianum* on intact *Arabidopsis* plants. **A**, Four-week-old plants were inoculated with *C. higginsianum* (left panel) and *C. linicola A1* (right panel) and photographed at 4 days postinoculation (dpi). Arrowheads show inoculation sites. **B**, Trypan blue staining showed the infection pattern of *C. higginsianum* (left panel) and *C. linicola A1* (right panel) in intact plants. The microscopic images were taken at 4 dpi; ap, appressorium; ph, primary hyphae; sh, secondary hyphae. Scale bar = 10 µm.

Fig. 4. Resistance-gene-mediated defense responses in detached leaves. **A**, Disease symptom development on intact plants (upper panel) and detached leaves (lower panel) of ecotypes Col-0 and Eil-0. The leaves were inoculated with droplets of *Colletotrichum higginsianum* conidial suspension and photographed at 5 days postinoculation (dpi). **B**, Cytochemical examination of fungal development in intact plants (upper panel) and detached leaves (lower panel) of ecotypes Col-0 and Eil-0. The leaves were inoculated with droplets of *C. higginsianum* conidial suspension and were stained with trypan blue at 3 dpi; ap, appressorium; ph, primary hyphae; sh, secondary hyphae. Scale bar = 10 µm. **C**, Infection-associated callose deposition on detached Eil-0 leaves at 3 dpi with *C. higginsianum*. Aniline blue stained leaves were examined under epifluorescent (right panel) or light (left panel) microscope, respectively. Arrows indicate appressoria and arrowhead indicates an infection vesicle underneath the penetration site. Scale bar = 10 µm. **D**, Infection-associated H_2O_2 production on detached Eil-0 leaves. The Col-0 (left panel) and Eil-0 (right panel) leaves were inoculated with droplets of *C. higginsianum* conidial suspension and infiltrated with 3,3'-diaminobenzidine at 3 dpi. Dotted lines indicate the boundary between the inoculated and noninoculated areas. Scale bar = 10 µm.

persensitive response, whereas the symptoms on detached Eil-0 leaves, similar to Col-0 leaves, showed necrotic water-soaked lesions (Fig. 4A), suggesting that the *R*-gene-mediated defense mechanism was compromised in detached Eil-0 leaves. Examination of fungal growth revealed that no penetration occurred in attached Eil-0 leaves, but successful penetrations in detached Eil-0 leaves were comparable with those seen on detached Col-0 leaves (Fig. 4B). Penetration in detached Eil-0 leaves was directly associated with secondary hyphae. Unlike in intact Col-0 plants, where abundant *C. higginsianum* primary hyphal growth accompanies infection, intact Eil-0 plants harbored very few primary hyphae (Fig. 4B). Interestingly, hypersensitive cell death-associated callose deposition (Fig. 4C) and reactive oxygen species (ROS) accumulation (Fig. 4D) were still evident in detached Eil-0 leaves. These results show that *R*-gene-mediated resistance also can be altered following leaf detachment. The detached Eil-0 leaf fails in penetration resistance against *C. higginsianum* attack but retains its ability to produce a hypersensitive reaction and ROS burst. The hypersensitivity of infected cells leads to the conversion from biotrophy to necrotrophy of *C. higginsianum* immediately after penetration. Our results also support that basal

penetration resistance could be uncoupled from the *R*-gene-mediated hypersensitive response and ROS generation.

Disease susceptibility and defense responses in detached leaves are uncoupled from SA-dependent signaling pathways but associated with ET perception.

To investigate whether defense signaling pathways are altered in detached leaves, we inoculated a series of *Arabidopsis* defense mutants with virulent *C. higginsianum* to follow disease progress in both attached and detached leaf assays. Nine mutant or transgenic plants were selected to represent the well-characterized SA- and ET/JA-dependent defense pathways, including four SA-related mutants (*npr1-3*, *NahG*, *sid2-1*, and *pad4-1*), three ET-insensitive mutants (*ein2-1*, *ein3-1*, and *etr1-1*), and one JA-insensitive mutant (*jar1-1*).

First, the responses of these mutants to *C. higginsianum* were investigated using an intact plant assay. All inoculated plants developed disease symptoms 3 dpi, but the severity varied significantly among the mutants after longer periods of incubation (Fig. 5A). In general, the plants impaired in the SA pathway exhibited more severe symptoms, among which *NahG*,

Fig. 5. Pathogenicity of *Colletotrichum higginsianum* (*C.h.*) on both detached and attached leaves of *Arabidopsis* mutants with impaired defense pathways. **A**, Disease symptoms on intact plants. The wild-type Col-0 and different mutant plants were inoculated with droplets of conidial suspension and photographed at 10 days postinoculation (dpi). **B**, Northern blot analysis for defense gene expression. Total RNA (10 µg) extracted from the whole plants of wild-type Col-0 and mutants with or without *C. higginsianum* infection was hybridized with *PR1* and *PDF1.2*, respectively. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. **C**, Disease symptom development on detached leaves. The leaves from wild-type Col-0 and different mutants were inoculated with droplets of conidial suspension and photographed at 6 dpi.

Fig. 6. Pathogenicity of *Colletotrichum higginsianum* on intact plants and detached leaves of ethylene-insensitive mutant *ein2-1*. **A**, Comparison of disease symptom (upper panels) and hypha development (lower panels) on intact plants between wild-type Col-0 and *ein2-1*. The plants were sprayed with conidia suspensions and photographed at 4 days postinoculation (dpi). Arrowheads show the difference between extended biotrophic primary hyphae (Col-0) and quick transition to necrotrophic secondary hyphae (*ein2-1*) at 3 dpi. Scale bar = 20 μ m. **B**, Comparison of disease symptom (upper panels) and hyphal development (lower panels) on detached leaves between wild-type Col-0 and *ein2-1*. The detached leaves were sprayed with conidia suspensions and photographed at 5 dpi. Arrowheads indicate the appearance of abundant necrotrophic secondary hyphae in Col-0 and only biotrophic hyphae in *ein2-1* at 4 dpi. Scale bar = 20 μ m. **C** and **D**, Northern blot analysis for defense gene expressions in **C**, intact plants and **D**, detached leaves, respectively. Total RNA (10 μ g) extracted from the whole plants of wild-type Col-0 and *ein2-1* with (*C.h.*) or without (Mock) *C. higginsianum* inoculation was hybridized with *PR1* and *PDF1.2*, respectively. Equal loading of RNA was monitored by gel staining with ethidium bromide.

Fig. 7. Correlation between disease symptom development and senescence on detached leaves. **A**, Northern blot analysis for senescence-associated gene expression in detached-leaves of Col-0 and *ein2-1* during *Colletotrichum higginsianum* infection. Total RNA (6 μ g) extracted from wild-type Col-0 and *ein2-1* leaves at 1 to 5 days with or without pathogen inoculation was hybridized with senescence-associated genes *SAG13*, *SAG21*, *SAG101*, *SEN1*, *SEN4*, and *CAB1*, respectively. RNAs isolated from intact plants before detachment are an additional control. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. **B**, Effect of different plant hormones on the disease symptom development. The detached leaves of Col-0 were inoculated with *C. higginsianum* and co-incubated with water (CK) or with methyl jasmonate (MeJA, 200 μ M), Ethephon (200 μ M), salicylate (SA, 200 μ M), Zeatin-R (2 mg/liter), or Kinetin (2 mg/liter). The disease symptoms were photographed at 3 days postinoculation (dpi) (upper panel) and 6 dpi (lower panel). **C**, Disease symptom development on senescence-accelerating autophagy mutants. The detached leaves of wild-type Ws-0 and mutant *atg4a/4b* and *atg9-1* were inoculated with *C. higginsianum*, and photographed at 3 dpi. The leaves were arrayed according to developmental stages of a single plant from younger to older (1 to 7).

sid2-1, and *pad4-1* were more dramatic compared with the wild-type Col-0. Plants deficient in ET signaling (*ein2-1*, *ein3-1*, and *etr1-1*) exhibited slightly more severe disease than the wild type. JA-insensitive *jar1-1* plants had a disease phenotype similar to the wild type. Meanwhile, the defense gene expression in mutant and wild-type plants before and after pathogen inoculation was monitored (Fig. 5B). The result not only confirmed that defense gene expression, regulated by both SA (*PR1*, a marker for SA pathway) and ET/JA (*PDF1.2*, a marker for ET/JA pathway) pathways, was activated by *C. higginsianum* infection, but also showed compromised responses in the corresponding mutants, as expected. Basal expression of defense genes before inoculation also was evident in some of the mutants, which has been suggested to contribute to the antagonistic effects between SA and ET/JA pathways (Dong 2001; Ferrari et al. 2003; Feys et al. 2000; Kunkel and Brooks 2002).

When compared with the results in intact plants, detached-leaf assays using the same set of mutants showed altered results (Fig. 5C). All mutants exhibited similar or less disease symptom severity compared with wild-type Col-0. Remarkably, the ET signaling-defective mutants *ein2-1* and *ein3-1* exhibited a resistant-like reaction on detached leaves. The results indicate that pathogenicity variations between attached and detached leaves are common in *Arabidopsis-Colletotrichum* spp. interactions, and leaf detachment uncouples anthracnose symptom development from common defense signaling pathways.

To better understand the contrasting disease severity between detached leaves and intact plants of the ET-defective mutants after inoculation with *C. higginsianum*, we further examined this change in detail using *ein2-1* with a spray inoculation method. First, we compared disease symptoms in intact plants of Col-0 and *ein2-1* (Fig. 6A). The *ein2-1* plant exhibited more severe disease symptoms than Col-0 (Fig. 6A, upper panels, showing macroscopic lesions already at 3 dpi), and microscopic examination of fungal development revealed that the transition from primary hyphae to secondary hyphae was much faster in *ein2-1* than in wild-type plants (Fig. 6A, lower panels). Conversely, the detached *ein2-1* leaves showed less chlorosis and smaller lesions compared with Col-0 (Fig. 6B, upper panels). Cytological examination confirmed that fungal development also was retarded in detached *ein2-1* leaves compared with Col-0 (Fig. 6B, lower panels). In *ein2-1* leaves, the primary hyphal stage was retained more than 24 h longer than that in wild-type leaves. Northern blot analysis verified that expression of both *PR1* and *PDF1.2* was compromised in intact *ein2-1* plants after *C. higginsianum* infection, and this was more conspicuous for *PDF1.2* (Fig. 6C). However, induced expression of these defense genes was less significant in detached leaves of both the wild type and *ein2-1* (Fig. 6D), and there was no correlation between disease severity and level of defense gene expression. Taken together, these results suggest that disease symptom development is uncoupled from expression of defense-responsive genes in detached leaves, but is associated with ET perception.

Pathogenicity on detached leaves is associated with leaf senescence.

Disruption of genes involved in ET synthesis or perception is well known to postpone senescence (Broekaert et al. 2006; Chao et al. 1997; van Loon et al. 2006). Thus, we speculated that the reduced disease severity on detached *ein2-1* leaves was related to the suppression of senescence. To test this hypothesis, we examined senescence-associated gene (*SAG*) expression in detached leaves of *ein2-1* and wild-type *Arabidopsis* plants, with or without *C. higginsianum* inoculation (Fig. 7A). Expression of *SAGs*, including *SAG21* (Weaver et al.

1998), *SAG101* (He and Gan 2002), *SEN1* (Oh et al. 1996), and *SEN4* (Park et al. 1998), was induced by *Colletotrichum* spp. infection on wild-type leaves, especially at 3 to 5 dpi. This pathogen-induced expression was weaker in the *ein2-1* mutant than in Col-0, indicating that ET insensitivity prevents leaf senescence stimulated by these treatments. *SAG 13* (Weaver et al. 1998) did not show significant induction. Expression of *CABI* (Leutwiler et al. 1986), a negative indicator of senescence, was downregulated upon leaf detachment and pathogen infection in both the wild type and *ein2-1*.

To verify the dependence of symptom development on accelerating senescence in detached leaves, we induced leaf senescence by exogenously applying plant hormones to detached Col-0 leaves (Fig. 7B). Senescence was accelerated in leaves treated with the senescence-promoting hormones methyl jasmonic acid (MeJA), Ethephon, and SA (Morris et al. 2000), as was disease symptom development compared with untreated leaves. Conversely, in leaves treated with Zeatin-R and Kinetin, hormones that inhibit senescence, milder disease symptoms were observed (Fig. 7B).

To further validate the correlation between senescence and symptom development, we employed *Arabidopsis* autophagy (ATG, also named APG) mutants. Autophagy is a complex intracellular process for vacuolar bulk degradation of cytoplasmic components, and has emerged as an important pathway for regulating leaf senescence (Hanaoka et al. 2002; Xiong et al. 2005; Yoshimoto et al. 2004). As anticipated, detached leaves of both ATG null mutants *atg4a/4b* and *atgap9-1* exhibited more severe disease symptoms after inoculation than their parental wild-type Wassilewskija (Ws-0) regardless of leaf age (Fig. 7C). Taken together, these results suggest that senescence plays a significant role in anthracnose symptom development in detached *Arabidopsis* leaves.

DISCUSSION

The adaptation of *C. linicola* A1 on *Arabidopsis*.

Many *Colletotrichum* spp. have evolved a hemibiotrophic strategy for plant colonization (Perfect et al. 1999). Among the infection structures, the melanized appressoria are essential for penetration by generating the high turgor pressure necessary for mechanical penetration through the plant cuticle and cell wall (Bechinger et al. 1999). In our study, we found that all of the test isolates can form melanized appressoria on detached *Arabidopsis* leaves, despite variations in shape, size, or timing of the formation. However, only *C. linicola* A1 could readily penetrate the host cell wall and develop intracellular hyphae, suggesting that pathogenesis is terminated during the switch from surface to invasive growth for these nonadapted *Colletotrichum* spp. (Shimada et al. 2006). It was proposed previously that this kind of host resistance was governed by an inducible immune response rather than constitutive preformed barriers, because actin filament function and coincident induction of papillary callose were required (Shimada et al. 2006). This notion is consistent with a report that nonpathogenic *C. lindemuthianum* triggered progressive defense responses in bean plants during appressorium-mediated penetration (Veneault-Fourrey et al. 2005). In contrast to nonadapted *Colletotrichum* spp., the adapted pathogen *C. higginsianum* might evade the formation of papillary callose through faster penetration-peg formation and entry, or suppress the induced basal defense pathways through an as yet unknown mechanism.

The failure of pathogenic fungi to breach the plant cell wall constitutes a major component of immunity in nonhost species (species outside the pathogen host range) and accounts for a proportion of aborted infection attempts on susceptible host plants (basal resistance) (Collins et al. 2003; Greenshields et

al. 2004). Initially, we considered that *C. linicola* A1 might be a well-adapted strain on *Arabidopsis* Col-0, due to abundant appressorium formation and faster development of both primary and secondary hyphae than those of virulent *C. higginsianum* in detached leaves. However, the pathogenicity tests on intact plants countered this assumption. It appears that the detachment-associated physiological and molecular state changes of the leaf, such as initiation of ET-dependent senescence and inactivation of defense pathways, which are discussed below, contributes to the colonization of *Arabidopsis* by this fungus. It would be interesting to compare similarities between this isolate and quiescent *Colletotrichum* spp., which remain dormant on the colonized tissue surfaces until the tissues mature or senesce (Latunde-Datta 2001; Prusky and Lichter 2007; Prusky et al. 2000). Our results suggest that the interaction between *Colletotrichum* spp. and *Arabidopsis* is complex and that, as a hemibiotroph, the genus *Colletotrichum* could be an excellent model to study the evolution of pathogenicity.

Distinct defense responses in detached and attached leaves of *Arabidopsis*.

Although the majority of disease tests are evaluated on the whole-plant level, large- or small-scale screenings or assays using detached plant tissues commonly are employed for necrotrophic (Chen et al. 2006; Kagan and Hammerschmidt 2002; Veronese et al. 2006; Zheng et al. 2006) and hemibiotrophic pathogens (Coca et al. 2004) and, occasionally, obligate biotrophic (Girgi et al. 2006) pathogens, and these results subsequently are applied to intact plants. In this study, we present contrasting results using an *Arabidopsis*-*Colletotrichum* spp. pathosystem, adding a layer of complexity and caution for research that aims to study defense or pathogenicity using detached leaves only.

It remains unclear whether the observed differences between attached and detached leaf assays are unique to this hemibiotrophic pathogen. It is generally believed that SA-controlled processes provide protection against biotrophic pathogens, whereas JA/ET pathways are more important against necrotrophic pathogens (Glazebrook 2005). Due to a mutually inhibitory effect between the SA and JA/ET pathways, defense mechanisms against biotrophs and necrotrophs might likewise be antagonistic. However, because hemibiotrophic *Colletotrichum* spp. possess both biotrophic and necrotrophic life stages, it seems reasonable to suspect a mixed defense response to this pathogen. Indeed, our study demonstrated that a disturbance of genes associated with either the SA or ET pathway compromised resistance in intact plants to *C. higginsianum*. Notably, the SA-pathway mutants (especially *pad4*, *NahG*, and *sid2*) were more susceptible than ET-pathway mutants. The uneven contribution of the two defense pathways to disease outcome could be because the biotrophic stage of *C. higginsianum* is critical to initiate SA-mediated defense responses, or that the antagonistic effect of the SA pathway overrules the ET pathway when both are activated simultaneously in intact plants.

The expression of pathogenesis-related (PR) genes, such as *PR1* and *PDF1.2*, is a hallmark of the activation of SA-dependent or JA/ET-dependent defenses, respectively (Thomma et al. 1998, 2001; Van Loon and Van Strien 1999). In this study, induction of both *PR1* and *PDF1.2* genes was found after inoculation of intact plants with *C. higginsianum*, again providing evidence that both SA and JA/ET pathways were activated for defense against hemibiotrophs. Our results are consistent with a report by Narusaka and associated (2004), in which they used the same pathosystem and found that the JA/ET-responsive *PR-4* and *PDF1.2* and SA-dependent *PR-2* and *PR-5* were induced, but the authors did not provide information on the more widely studied *PR1* gene.

In contrast to the results of the intact-plant assays, defense gene expression in detached leaves showed only marginal induction after inoculation with *C. higginsianum*. Our results show that common defense systems mediated by the SA and JA/ET pathways are less responsive in detached leaves than in intact plants, and that this suppression of defense pathways in detached leaves could be crucial for the successful infection of nonadapted pathogens. In this scenario, we hypothesize that compromised induction of SA- and ET/JA-dependent pathways contributes to successful penetration and aggressive colonization in detached *Arabidopsis* leaves by *C. linicola* A1. This assumption is further supported by an alternative observation that penetration resistance on ecotype Eil-0 also was compromised in detached leaves, although the hypersensitive response and oxidative burst were still evident. Eil-0 previously was reported to possess an *R*-gene-like locus, *RCH1*, and was highly resistant to *C. higginsianum* during intact-plant assays (Narusaka et al. 2004). Apparently, both *R*-gene-associated and SA- and ET/JA-dependent defense pathways are altered in detached leaves. However, biochemical and molecular mechanisms underlying the loss of a primary defense response in detached leaves likely is associated with penetration resistance, although this remains to be fully elucidated.

The role of ET in defense responses to *Colletotrichum* spp.

The phytohormone ET is a modulator of many aspects of plant life, including various mechanisms by which plants react to pathogen attack (Broekaert et al. 2006; van Loon et al. 2006). Induced ET biosynthesis and subsequent intracellular signaling through a single conserved pathway are well characterized (Alonso and Stepanova 2004; Guo and Ecker 2004). Pathogen-induced endogenous ET or exogenously applied ET has been shown to increase defense-related gene expression. However, the role of ET in determining the outcome of plant-pathogen interactions is still not clear, and even controversial (van Loon et al. 2006). For example, mutations in the central regulatory gene *EIN2*, encoding an NRAMP-like signal transduction-associated metal ion transporter (Alonso et al. 1999), lead to increased susceptibility to the necrotrophic fungus *Botrytis cinerea* and necrotrophic bacterium *Erwinia carotovora*, but shows no alteration in response to the biotrophic bacterium *Pseudomonas syringae* pv. *tomato*, the oomycete *Peronospora parasitica*, or the necrotrophic *Alternaria brassicicola* (Lawton et al. 1995; Norman-Setterblad et al. 2000; Pieterse et al. 1998; Thomma et al. 1999). Even within a single pathosystem, the alteration in ET-dependent disease responses can be contradictory (Lund et al. 1998; van Loon et al. 2006). Nevertheless, the use of ET mutants and transgenic plants has provided additional evidence to claim the positive role of ET in defense response, especially to necrotrophic pathogens (Chen et al. 2003; Hoffman et al. 1999; Knoester et al. 1998). The results from our intact-plant assay showed that ET-insensitive mutants exhibited compromised defense responses to hemibiotrophic *C. higginsianum*, which supports the positive role of ET pathway in disease resistance.

In detached leaves, the role of the ET perception in anthracnose development is negative and appears to be a susceptibility factor. In the ET signaling-defective mutants, disease development by both adapted *C. higginsianum* and near-adapted *C. linicola* A1 was much slower than that on wild-type and other defense-impaired leaves, implying that ET-dependent and defense (*PDF1.2*)-independent physiological processes (e.g., senescence) are required for disease symptom development on detached leaves. ET is well known to promote chlorophyll degradation and tissue senescence (Stall and Hall 1984), which correlates with chlorotic disease symptoms (surrounding the maceration area but not containing pathogen). In our *Arabi-*

dopsis-Colletotrichum spp. assays, we found that chlorosis was obvious in all of the detached leaves after pathogen inoculation except those of ET-insensitive mutants. ET-dependent senescence does not seem to interfere with pathogen penetration resistance or invasion success on detached leaves, but does affect the post-invasion process and symptom development, although ET has been reported to regulate the expression of cell wall components such as extensin (Showalter 1993). Together, our results show that ET-associated senescence plays a major role in anthracnose symptom development in detached *Arabidopsis* leaves.

Interplay between senescence and anthracnose disease.

In this study, we present evidence suggesting that senescence, in combination with ET, is a major factor for *Arabidopsis* susceptibility to *Colletotrichum* spp. on detached leaves: first, the symptom development is faster in older leaves than in younger leaves of the same plant; second, ET-insensitive mutants with retarded senescence exhibit the slowest disease symptom development; third, artificially applying senescence-promoting or -inhibiting agents to leaf tissues can correspondingly enhance or reduce disease symptom development; and fourth, genetically modified senescence mutants exhibit more rapid disease symptom development compared with wild-type plants. The correlation between senescence and disease symptom development is consistent with previous reports from other *Colletotrichum* spp. Leaf senescence of round-leaved mallow affected the biotrophic infection phase of *C. gloeosporioides* (Wei et al. 1997). Furthermore, Zulfiqar and associates (1996) showed that *C. gloeosporioides* did not penetrate healthy citrus leaves, whereas the pathogen still was able to infect and colonize detached citrus leaves that had been induced to senesce.

Colletotrichum pathogens often undergo an inactive infection stage as biotrophs after landing on the host surface (quiescent or latent stage), but activate their infection during host ripening and senescence (Latunde-Data 2001; Prusky and Lichter 2007; Prusky et al. 2000). This latency is an important phenomenon for postharvest disease development. The mechanisms of transition from biotrophy to necrotrophy are proposed to be related to factors that are modulated at the intracellular level and that are affected by nutrients and the ambient pH (Prusky and Lichter 2007). Compounds secreted by the pathogen, such as organic acids or ammonia, have a significant effect on host responses and can play critical physiological roles in the initiation of necrotrophic development. For instance, the ammonification at the edge of the *Colletotrichum* infection site may lead to deregulation of host responses that, in turn, could facilitate the activation of pathogenicity genes, although the identity of signals that induces the transition is not yet known (Prusky and Lichter 2007). Our findings in detached *Arabidopsis* leaves show that detached tissues become not only naturally more senescent but also less defensive. It will be interesting to investigate how senescence-accompanying physiological changes such as pH or sugar content are interlinked with defense pathways.

MATERIALS AND METHODS

Plant materials.

Arabidopsis accessions Col-0, Ws-0, and Eil-0 and their corresponding mutants were grown and managed routinely in a growth chamber as described (Liu et al., 2005a). The following mutants or transgenic plants were used in this study: *npr1-3* (Cao et al. 1997), *sid2-1* (Nawrath and Metraux 1999), *pad4-1* (Glazebrook et al. 1997), *ein2-1* (Roman et al. 1995), *ein3-1* (Chao et al. 1997), *etr1-1* (Chang et al. 1993), *jar1-1* (Staswick et al. 2002), *NahG* (Lawton et al. 1995), *atg4a/4b*

(Ws-0 background) (Yoshimoto et al. 2004), and *atapg9-1* (Ws-0 background) (Hanaoka et al. 2002). All mutants are derived from the Col-0 background except as noted.

Pathogen inoculations.

Two species of *Colletotrichum* were used in this study. The isolate *C. linicola* A1 was identified by screening pathogenicity of a *Colletotrichum* collection on *Arabidopsis* detached leaves. Twenty *Colletotrichum* isolates, all of which were collected by Agriculture and Agro-Food Canada, Saskatoon, Saskatchewan, from various hosts, including alfalfa (*Medicago sativa*), chamomile (*Matricaria perforata*), lentil (*Lens culinaris*), field bindweed (*Convolvulus arvensis*), mallow (*Malva pusilla*), red clover (*Trifolium pretense*), and soybean (*Glycine max*), were screened. Only the isolate 93-121 A1 (defined as *Colletotrichum linicola* A1) from field bindweed caused macroscopic disease symptoms and was used for further study. The species *C. higginsianum* Sacc. (IMI349061), originating from *Brassica rapa*, was obtained from CABI Bioscience (Egham, Surrey, U.K.). The fungi were propagated at room temperature on V8 medium as described previously (Wei et al. 2004).

For intact-plant assays, 4 to 5-week-old plants either were sprayed with conidial suspensions (1×10^6 spores ml⁻¹ in distilled water) or spotted with 5- to 10- μ l droplets on the leaf surface on either side of the leaf midvein. The plants were kept in a 100% humidity chamber immediately after inoculation (Narusaka et al. 2004; O'Connell et al. 2004). Detached leaves were inoculated in petri dishes (with wet filter paper inside) using the same methods but maintained with a 12-h photoperiod at a light intensity of 30 μ E m⁻² s⁻¹. The disease symptoms were evaluated by the speed of chlorosis and tissue maceration and by lesion size (Wei et al. 2004). All the attached and detached leaf assays were performed at least twice.

Cytology of fungal development and detection of callose and H₂O₂.

Infected leaves were harvested at varying time points and fixed in a fixation solution (60% methanol, 30% chloroform, and 10% acetic acid) until required. After rehydration through an ethanol gradient, the tissue was stained with trypan blue for fungal detection or aniline blue for callose deposition and examined by light or epifluorescence microscopy, respectively. Acervuli were observed using scanning electron microscopy. Functional integrity of the plasma membrane and tonoplast of infected leaf epidermal cells were evaluated by treating the infected tissue with 0.8 M NaNO₃ (pH 8.0). For in situ histochemical detection of H₂O₂, the inoculated leaves were infiltrated with 3,3'-diaminobenzidine at 1 mg ml⁻¹ for 3 to 5 h and then cleared by fixation solution as above.

Treatment of plant leaves with phytohormones.

Detached leaves of 4- to 5-week-old Col-0 plants were placed on filter paper presoaked with MeJA (200 μ M), Ethephon (200 μ M), SA (200 μ M), Zeatin-R (2 mg liter⁻¹), or Kinetin (2 mg liter⁻¹) and inoculated with droplets containing 1×10^6 *C. higginsianum* conidia ml⁻¹. H₂O-treated leaves were used as controls. The treatment were performed twice and obtained similar result. All chemicals were purchased from Sigma-Aldrich (St. Louis), except for MeJA (Bedoukian Research, Danbury, CT, U.S.A.).

RNA gel blot analysis.

Arabidopsis leaves with or without inoculation were collected at different intervals, flash frozen in liquid nitrogen, and stored at -80°C until required. Total RNA extraction from control or treated leaves as well as Northern blot analysis was performed as described (Liu et al. 2005b). Probes, consisting of the entire

or partial coding region for *PR-1* (At2g14610), *PDF1.2* (At5g44420), *SAG13* (At2g29350), *SAG21* (AT4G02380), *SAG101* (At5g14930), *SEN1* (At4g35770), *SEN4* (At4g30270), and *CABI* (At1g29930), were amplified by reverse-transcription polymerase chain reaction (PCR) using gene-specific primer pairs, at least one of which was specific for 3' or 5' untranslated regions, or by PCR from cloned plasmids. The oligo sequences of these primers are shown in Supplementary Table S2. All RNA gel blot analyses were conducted with at least two biological replications.

Sequencing and analysis of rDNA-ITS region.

DNA extraction from fungal mycelia and sequencing were performed as described (Moriwaki et al. 2002). Primers of fungal rDNA-ITS regions ITS-F 5'-tggaagtaaaagtcgtaacaagg and ITS-R 5'-tcctccgcttattgatatgc were used for amplification (White et al. 1990). The ITS sequences for reference isolates of *Colletotrichum* spp. were obtained from the GenBank database. The alignment of ITS1 sequences and construction of phylogenetic tree were performed online using ClustalW.

ACKNOWLEDGMENTS

Arabidopsis seed of the wild types and different mutants used in this study were obtained from The Arabidopsis Biological Resources Center, Ohio State University. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant to Y. Wei. We are grateful to Y. Ohsumi (National Institute for Basic Biology, Japan) for providing *atg4a/4b* and *atg9-1* mutants and P. Fobert (National Research Council of Canada, Plant Biotechnology Institute) for providing *NahG* seeds. We also thank J. Feng, X. Wu, and N. Bhuiyan for technical assistance.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information GenBank database:
www.ncbi.nlm.nih.gov
 ClustalW Software: www.ebi.ac.uk/clustalw