RCH1*, a Locus in *Arabidopsis* That Confers Resistance to the Hemibiotrophic Fungal Pathogen *Colletotrichum higginsianum

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When challenged with the crucifer pathogen *Colletotrichum higginsianum*, *Arabidopsis thaliana* ecotype Columbia (Col-0) was colonized by the fungus within 2 to 3 days, developing brown necrotic lesions surrounded by a yellow halo. Lesions spread from the inoculation site within 3 to 4 days, and subsequently continued to expand until they covered the entire leaf. Electron microscopy confirmed that *C. higginsianum* is a hemibiotroph on *Arabidopsis*, feeding initially on living cells as a biotroph before switching to a necrotrophic mode of growth. A collection of 37 ecotypes of *Arabidopsis* varied in their responses to infection by *C. higginsianum*. The ecotype Eil-0 was highly resistant, with symptoms limited to necrotic flecking and with only very limited fungal colonization. Analyses suggested that the hypersensitive response and reactive oxygen species may be important in this defense response. Expression analyses with cDNA microarrays indicated that the defense reaction depends primarily on the jasmonic acid- and ethylene-dependent signaling pathways and, to a lesser extent, on the salicylate-dependent pathway. Crosses between the Eil-0 and Col-0 ecotypes suggested that the resistance in Eil-0 was dominant and was conferred by a single locus, which we named *RCH1*. *RCH1* is the first resistance locus to be identified from *Arabidopsis* against the hemibiotrophic fungus genus *Colletotrichum*.

In nature, plants interact with many different types of microbes, but only a few of these actually damage the plants. Plant diseases occur only rarely 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. These responses include rapid localized cell death, known as the hypersensitive response (HR) (Morel and Dangl 1997), cross-linking of cell wall proteins (Bradley et al. 1992), phytoalexin formation (Bailey and Mansfield 1982), and the production of other antimicrobial secondary metabolites and pathogenesis-related proteins (PR proteins) (Linthorst 1991; Van Loon 1985), many of which also exert antimicrobial properties. Signaling molecules implicated in these inducible defense systems include salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and reactive oxygen species (ROS) (Hammond-Kosack and Jones 1996; Ryals et al. 1996). In a compatible interaction, a virulent pathogen is able to defeat these plant defense mechanisms, either by avoiding detection or by neutralizing host defenses.

A major goal in plant pathology is to understand the molecular basis of specificity in plant–pathogen interactions. A crucial event is the recognition of the invading pathogen by the plant at an early stage of infection. Specific recognition is thought to be mediated through direct or indirect interactions between the product of a resistance (*R*) gene in the plant and the product of a corresponding avirulence (*avr*) gene in the pathogen. Recently, *Arabidopsis thaliana* has been studied intensively as a model host plant for diseases caused by bacterial, fungal, and viral pathogens. *A. thaliana* provides a genetically amenable system in which to examine the various components of disease resistance (Koch and Slusarenko 1990). Many accessions (ecotypes) of *A. thaliana* are available, which differ genetically as a result of selection pressures imposed by their different environments of origin (Kagan and Hammerschmidt 2002). These differences include variation in resistance to pathogens. For example, different ecotypes of *Arabidopsis* differed 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), the bacterium *Xanthomonas campestris* pv. *campestris* (Tsuji et al. 1991), and the oomycetes *Albugo candida* (Holub et al. 1995)

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and *Peronospora parasitica* (Mauch-Mani et al. 1993). Resistance to these pathogens is due to the presence of one or more *R* genes. The types of *R* genes varied among ecotypes, which may be a reflection of variations in disease pressures in the locations where the ecotypes were collected (Kagan and Hamerschmidt 2002).

Colletotrichum spp. cause anthracnose diseases that typically appear as small or large dark-colored spots or slightly sunken lesions on the foliage, stems, or fruit of a wide range of important crop and ornamental plants (Agrios 1988). Species of this genus have been used for many years in studies concerning fungal differentiation and fungal-plant interactions (Bailey et al. 1992). During the colonization of plant hosts, most fungal pathogens exhibit one of two modes of nutrition: biotrophy, in which nutrients are obtained from living host cells, and necrotrophy, in which nutrients are obtained from host cells which previously have been killed by the fungus (Thrower 1966). However, species in the genus *Colletotrichum*

typically are hemibiotrophs, and utilize both of these nutritional strategies in turn. During their interactions with their hosts, *Colletotrichum* fungi develop a series of specialized infection structures, including germ tubes, appressoria, intercellular hyphae (IH), and secondary necrotrophic hyphae. A number of molecular approaches have been employed to study events involved in *Colletotrichum* spp. developmental biology and pathogenicity (Perfect et al. 1999). However, the host plants of the most commonly studied species of *Colletotrichum* have not themselves been good models for molecular analyses of the host side of the interaction.

Colletotrichum higginsianum was reported to cause typical anthracnose lesions on the leaves, petioles, and stems of turnip, mustard, and Chinese cabbage (Higgins 1917). Inoculation of leaves of the *A. thaliana* ecotype Columbia (Col-0) with *C. higginsianum* resulted in fungal growth, and disease symptoms reminiscent of those incited in these other cruciferous plants inoculated under the same conditions. In this study,

Fig. 1. Infection phenotypes of *Arabidopsis* leaves inoculated with the fungus *Colletotrichum higginsianum*. Leaves of 28- to 30-day-old plants were inoculated by placing between two and six 5- μ l drops of a spore suspension of *C. higginsianum* (5×10^5 spores ml^{-1}) on each leaf. Control plants (Col-0) were inoculated with distilled water instead of the fungus. Lesion phenotypes at 6 days postinoculation (dpi) are shown. Leaves were harvested at 6 dpi and stained with trypan blue. Arrows indicate the inoculation sites and lesions. Each picture shows a representative of three independent experiments.

Table 1. Interaction phenotypes between ecotypes of *Arabidopsis thaliana* and *Colletotrichum higginsianum* (MAFF305635)

<i>A. thaliana</i> ecotype	Origin	Disease reaction score	<i>A. thaliana</i> ecotype	Origin	Disease reaction score
Aa-0	Germany	1	Kl-0	Germany	3
Br-0	Czechoslovakia	1	Ko-2	Denmark	3
Bsch-0	Germany	1	La-1	Poland	3
Cvi-0	Cape Verde Islands	3	Lan-0	U.K.	3
Col-0	U.S.A.	3	Le-0	Netherlands	3
Col-gl	U.S.A.	3	Ler	Germany	3
Di-G	France	3	Nos	Germany	3
Ei-2	Germany	1	OY	Japan	3
Ei-6	Germany	3	Pn-0	France	2
Eil-0	Germany	1	Ri-0	Canada	3
FK	Japan	3	RLD	Russia	3
Fl-3	Finland	1	St-0	Sweden	1
Fuk	Japan	3	Ts-2	Spain	3
Gr-1	Austria	1	TY	Japan	3
Hi-0	Netherlands	1	Wa-1	Poland	3
Hir-1	Japan	3	Ws	Russia	2
IK	Japan	3	Xxx-0	Spain	3
Kil-0	U.K.	3	Yo-0	U.S.A.	3
Kin-0	U.S.A.	3			

we describe variation in the interaction phenotypes among various ecotypes observed upon inoculation with *C. higginsianum*, and we also identify a single putative *R* locus that is associated with the different interaction phenotypes. The anthracnose disease caused on the model plant *Arabidopsis* by *C. higginsianum* provides a valuable new system for analysis of fungal pathogenicity factors as well as of host responses in a hemibiotrophic disease interaction.

RESULTS

Responses of various *Arabidopsis* ecotypes to inoculation with *C. higginsianum*.

Three different strains of *C. higginsianum* (MAFF305635, MAFF305968, and MAFF305970) caused anthracnose disease symptoms on *A. thaliana* Col-0 plants that were similar to those incited on turnip plants (*Brassica rapa* L. and *B. oleracea*). When challenged with the fungus, Col-0 plants developed brown necrotic lesions, surrounded by a yellow halo. These lesions had expanded from the inoculation site by 4 days post inoculation (dpi) and, subsequently, spread over the

entire leaf. The fungus sporulated on the host, and the fungus recovered from infected plant material was used to complete Koch's postulates. There were no obvious differences among the fungal strains in their ability to cause disease symptoms; therefore, one strain (MAFF305635) was chosen for further experiments.

To evaluate the range of disease reactions of *Arabidopsis* ecotypes to *C. higginsianum*, we devised disease reaction scores (DRS) based on numerical ratings of two components, including the extent of pathogen colonization of the host as evaluated by lactophenol-trypan blue staining, and the severity of host chlorosis/necrosis. Using this scoring method, a total of 37 *Arabidopsis* ecotypes obtained from various habitats around the world were evaluated for susceptibility to *C. higginsianum* (Fig. 1; Table 1). Most of the interactions observed with these ecotypes were compatible, with DRS scores equivalent to the Col-0 plants. However, examples of incompatible phenotypes were found following inoculation of ecotypes Aa-0, Br-0, Bscl-0, Ei-2, Eil-0, Fl-3, Gr-1, Hi-0, Pn-0, St-0, and WS. *Arabidopsis* ecotype Eil-0 (DRS 1) appeared to be highly resistant, developing only small necrotic flecks at the inocu-

Fig. 2. *Arabidopsis* cells inoculated with *Colletotrichum higginsianum*. The inoculation of *Arabidopsis* Col-0 and Eil-0 plants with *C. higginsianum* was performed on 28- to 30-day-old plants by placing two 5- μ l drops of a suspension of 2×10^6 spores ml^{-1} in distilled water on each leaf. Inoculated leaves were observed with an electron microscope to examine the fungal behavior. **A**, Intact epidermal cells and a mesophyll cell on a Col-0 leaf are shown. Bar = 1 μ m. **B**, **C**, and **D**, Col-0 leaves inoculated with *C. higginsianum* for 2 and 3 days. A penetration peg breached both epidermal cell walls and papillae (**B**, bar = 1 μ m). The epidermal cell was not degenerated by the invasion of fungus (**C**, bar = 3 μ m). Infection hyphae were observed in epidermal and mesophyll cells (**D**; bar = 5 μ m). **E** and **F**, Eil-0 leaves inoculated with *C. higginsianum* for 2 and 3 days. Penetration pegs invaded into papillae at 2 dpi (**E**, bar = 1 μ m). Degenerated infection hyphae were observed in collapsed epidermal cells. Infection hyphae showed degenerated organelles and apparent leakage of cytoplasmic matrix. Live mesophyll cells responded to the hyphae by forming papillae at 3 dpi (**F**, bar = 1 μ m). A, appressorium; DIH, degenerated infection hypha; EC, epidermal cell; ECW, epidermal cell wall; EPM, epidermal plasma membrane; IH, infection hypha; MC, mesophyll cell; N, nucleus; PA, papilla; PP, penetration peg. Each picture shows a representative of three independent experiments.

lated sites by 3 to 4 dpi that did not expand further (Fig. 1). *Arabidopsis* ecotypes with a more moderate level of resistance, such as WS (DRS 2), developed brown necrotic lesions at the inoculated sites, but subsequent expansion of these lesions was restricted in comparison with lesions formed on the Col-0 plants (Fig. 1).

To learn more about the nature of the interaction of *C. higginsianum* with Col-0 (susceptible) and Eil-0 (resistant), electron microscopy (EM) was performed. Spores of *C. higginsianum* germinated and formed melanized appressoria on the surfaces of Col-0 leaves. By the time penetration pegs began to emerge from the appressoria at 2 dpi, papillae already had formed in the spaces between the host cell walls and plasma membranes. The papillae were composed of membranous fragments embedded within amorphous electron-dense materials (Fig. 2B). However, the papillae did not form an effective barrier against the penetration pegs (Fig. 2B). Some of the epidermal cells invaded by the fungal pathogen appeared to be intact during early stages of infection (within 2 to 3 dpi) (Fig. 2C), whereas others were collapsed. The penetration pegs had a diameter of 0.5 μm and developed into infection hyphae with a diameter of 2 to 3 μm within the epidermal cells. The infection hyphae had a rich cytoplasm containing organelles and ribosomes (Fig. 2B and C). Subsequently, the mesophyll cell invasion commonly was observed in the susceptible accession Col-0 (Fig. 2D).

The formation of germ tubes, appressoria, penetration pegs, and infection hyphae was similar when *C. higginsianum* was inoculated onto leaves of the resistant ecotype Eil-0. Papillae were formed by infected epidermal cells at what appeared to be the same rate as in susceptible Col-0 leaves (Fig. 2E). Penetration pegs breached these papillae, again at a rate similar to that in Col-0 leaves, and developed into infection hyphae within the epidermal cells. A difference in the response of Eil-0 compared with Col-0 then became evident. Invaded epidermal cells of Eil-0 were collapsed, and the cytoplasm of the hyphae within the collapsed epidermal cells appeared to be highly vacuolated. When infection hyphae had contacted mesophyll cell walls, papillae had been formed by those mesophyll cells at the contact points (Fig. 2F). Mesophyll cells adjacent to the epidermal cells were intact, however, (Fig. 2F), and fungal hyphae were only rarely observed in these cells during early stages of infection (within 2 to 3 dpi). These results sug-

gest that resistance in Eil-0 may be mediated through a process of hypersensitive cell death. These visible defense responses in epidermal cells may play an important role in limiting invasion of mesophyll cells of Eil-0 by *C. higginsianum*.

ROS accumulation.

An oxidative burst giving rise to localized accumulation of ROS is an early event typically associated with HR in plants (Thordal-Christensen et al. 1997). We assayed production of ROS in Col-0 and Eil-0 plants at pathogen penetration sites to determine whether ROS accumulation could be involved in the defense response. Eil-0 plants did not produce a detectable level of H_2O_2 after challenge with *C. higginsianum* up until 2 dpi (Fig. 3). However, between 2 and 3 dpi, Eil-0 plants began to generate high levels of H_2O_2 (Fig. 3). Thus, production of H_2O_2 occurred within the same time frame as the apparent hypersensitive cell death response in the Eil-0 ecotype, as observed by EM (Fig. 2). In contrast, Col-0 plants produced no detectable H_2O_2 after challenge with *C. higginsianum*, even up until 4 dpi (Fig. 3). Similar results were obtained with various other *Arabidopsis* ecotypes, including Ler, Nos (compatible), and Bscl-0 (incompatible) (data not shown).

Camalexin induction.

The production of the *Arabidopsis* phytoalexin camalexin on Col-0 and Eil-0 plants was measured in response to infection by *C. higginsianum*. When challenged with the fungus, Col-0 plants did not produce detectable levels of camalexin up to 2 dpi, but produced the phytoalexin by 4 dpi (Fig. 4). Camalexin scarcely could be detected in the inoculated Eil-0 plants, even at 4 dpi.

The *pad3-1* mutant is derived from the Col-0 ecotype of *Arabidopsis* and it is unable to produce camalexin (Glazebrook and Ausubel 1994). As expected, inoculation of this mutant with *C. higginsianum* resulted in no detectable induction of camalexin synthesis up to 4 dpi (Fig. 4). The mutant was more susceptible to the pathogen than the wild-type Col-0 ecotype, developing much more severe lesions in response to inoculation at 6 dpi (Fig. 1). The *pad3* gene encodes a cytochrome P450 enzyme resembling an enzyme from maize that is involved in the production of indole-type phytoalexins (Zhou et al. 1999). We investigated expression of the *pad3* gene in Eil-0 and Col-0 leaves inoculated with *C. higginsianum*.

Fig. 3. H_2O_2 accumulation detected using 3,3-diaminobenzidine (DAB) staining in *Arabidopsis* Col-0 and Eil-0 leaves inoculated with *Colletotrichum higginsianum*. Leaves of 28- to 30-day-old plants were inoculated by placing two 5- μl drops of a spore suspension of *C. higginsianum* (5×10^5 spores ml^{-1}) on each leaf. H_2O_2 accumulation was detected by DAB staining at 2, 3, and 4 days postinoculation. Arrows indicate the H_2O_2 accumulation. A, appressorium; IH, infection hypha. Each picture shows a representative of three independent experiments using 10 leaves per time point. Bars = 20 μm .

sianum by using RNA gel blot analysis. The expression of the *pad3* gene in the Eil-0 plants was slightly induced at 2 dpi, but seemed to decrease again thereafter; whereas, in the Col-0 plants, expression was more strongly induced at 2 dpi, and then increased even further by 4 dpi (Fig. 5).

Induction of SA-, JA-, and ET-dependent defense responses by *C. higginsianum*.

We investigated induction of the pathogen-inducible genes *PR-2*, which encodes β -1,3-glucanase (Uknes et al. 1992); *PR-4*, which encodes a hevein-like protein (Potter et al. 1993); *PR-5*, which encodes thaumatin-like protein (Uknes et al. 1992); and *PDF1.2*, which encodes a plant defensin (Penninckx et al. 1996), in Eil-0 and Col-0 plants by using RNA gel blot analyses. *PR-2* and *PR-5* are markers for SA-dependent defense responses (Delaney et al. 1994; Penninckx et al. 1996; Thomma et al. 1999b), whereas *PR-4* and *PDF1.2* are indicators for JA- and ET-dependent responses (Penninckx et al. 1996, 1998; Thomma et al. 1998; Thomma et al. 1999b).

Expression of the *PR-4* gene in Eil-0 plants inoculated with *C. higginsianum* increased over time, with a peak at 2 dpi (Fig. 5). In the inoculated Col-0 plants, the expression of *PR-4* also increased with time; however, in contrast to the Eil-0 plants, expression continued to rise until 4 dpi. Transcript levels of *PDF1.2* increased between 0 and 4 dpi both in the Eil-0 and the Col-0 plants, but levels of accumulation at 4 dpi were much higher in the Col-0 ecotype. *PR-5* signals were relatively weak in both ecotypes, although it appeared that transcript quantities peaked at 4 dpi in Col-0 and at 2 dpi in Eil-0. *PR-2* transcripts could not be detected in either *Arabidopsis* ecotype during the course of this experiment. We also confirmed that the probe for *PR-2* actually was able to detect this gene product in these strains (data not shown).

To further investigate the roles of the SA-, JA-, and ET-dependent defense response pathways against *C. higginsianum*, we inoculated various *Arabidopsis* mutants with the pathogen. Mutant lines *eds5-1* and *eds16-1* do not produce SA (Dewdney et al. 2000; Nawrath and Metraux 1999); *nahG* fails to accumulate SA (Gaffney et al. 1993); *jar1-1* is insensitive to

JA (Staswick and Howell 1992); and *ein2-5* (Guzman and Ecker 1990) and *etr1-1* (Chang et al. 1993) are insensitive to ET. All of these mutants originally were derived from the Col-0 ecotype. At 6 dpi, the mutants were not noticeably different from the wild-type Col-0 plants in their response to the pathogen, as evaluated by our DRS (data not shown).

Microarray analysis.

To continue our investigation into the possible functions of SA, JA, and ET signaling in defense responses to *C. higginsianum*, we performed microarray analysis using arrays consisting of approximately 7,000 (7K) *Arabidopsis* full-length cDNAs. To assess the reproducibility of the microarray results, we conducted three independent analyses for each biological sample. The results demonstrated a high degree of correlation in fold change values between the different data sets (Table 2). After inoculation with *C. higginsianum*, the transcript levels of 120 genes were increased in Col-0 plants (Table 2; Fig. 6A). Treatment of Col-0 plants with SA, JA, and ET induced the expression of 178, 190, and 118 genes, respectively.

The ratios of expression levels of inoculated versus control Col-0 plants were $\log_2$ transformed and subjected to hierarchical clustering using GeneSpring (Silicon Genetics, San Carlos, CA) (Fig. 6A and B). The tendency was observed especially in 120 genes upregulated by the infection, and some genes were known to be important in JA- and ET-signaling pathways. They are ET responsive-element binding factors (*EREBs*) and hevein-like protein (*PR-4*), but most of the genes are unknown (Table 2). The 120 genes expressed in Col-0 plants inoculated with *C. higginsianum* were subjected to clustering by the inoculation and treatments (SA, JA, and ET). Results of cluster analysis of resultant expression profiles are shown in Figure 6A. The horizontal dendrogram of Figure 6A and the expansion figure, Figure 6B, indicate the relationship among experiments across all of 120 genes included in the cluster analysis. It was demonstrated that the expression profiles of genes highly expressed by inoculation with *C. higginsianum* resemble those of JA or ET treat-

Fig. 4. Accumulation of the *Arabidopsis* phytoalexin camalexin after inoculation of Col-0, Eil-0, and *pad3-1* plants with *Colletotrichum higginsianum*. The 28- to 30-day-old *Arabidopsis* Col-0, Eil-0, and *pad3-1* plants were inoculated with *C. higginsianum* and harvested after 1, 2, or 4 days. Data points represent averages with standard error of measurements on more than nine samples out of four to five independent experiments.

Fig. 5. Northern blot analysis of expression of *PR-2*, *PR-4*, *PR-5*, *PDF1.2* and *pad3* genes. The 28- to 30-day-old *Arabidopsis* Col-0 and Eil-0 plants were sprayed with a spore suspension of *Colletotrichum higginsianum* (5×10^5 spores ml^{-1}) and harvested 10 h or 1, 2, or 4 days later as indicated at top of the figure. As a control, leaf material also was collected just before inoculation (0). Each lane was loaded with 4 µg of total RNA. The DIG-labeled antisense RNA prepared from 300 bp upstream region from the 3' end of the full-length cDNA of *PR-2*, *PR-4*, *PR-5*, *PDF1.2*, and *pad3* genes was used as a probe.

Table 2. Expression profiles of 120 *Arabidopsis* genes significantly induced by inoculation with *Colletotrichum higginsianum*

Clone name	Time after inoculation (h)						MIPS ^b	Annotation
	10		24		48			
	Ratio ^a	<i>P</i> value	Ratio	<i>P</i> value	Ratio	<i>P</i> value		
Cell rescue, defense, cell death, and aging								
RAFL02-09-H01	3.3	0.04	8.0	0.01	15.3	0.05	At4g35770	Senescence-associated protein sen1
RAFL04-09-K06	7.4	0.00	1.4	0.07	0.9	0.83	At5g20630	Germin-like protein (GLP3b)
RAFL04-12-G16	1.3	0.57	2.2	0.07	9.6	0.29	At2g43570	Putative endochitinase
RAFL04-16-I15	1.7	0.17	2.2	0.02	17.2	0.01	At3g04720	Hevein-like protein precursor
RAFL05-09-M02	1.3	0.26	1.7	0.36	5.4	0.25	At5g06320	NDR1/HIN1-like protein 3 (NHL3)
RAFL05-19-P11	2.8	0.03	3.4	0.02	9.1	0.03	At3g10985	Wound-induced protein WI12
RAFL05-20-B01	2.7	0.04	6.2	0.18	10.7	0.21	At2g40000	Putative nematode-resistance protein
RAFL06-13-N20	3.1	0.15	4.3	0.14	24.0	0.06	At4g02380	Late embryogenesis abundant protein family
RAFL06-16-E18	3.2	0.00	6.5	0.19	5.4	0.04	At1g32920	Avr9/Cf-9 rapidly elicited protein 65
RAFL07-12-D22	1.7	0.02	2.2	0.11	5.4	0.10	At3g52430	Phytoalexin-deficient 4 protein (pad4)
RAFL08-13-M21	2.4	0.10	4.1	0.02	6.7	0.01	At3g10985	Wound-induced protein WI12
RAFL09-13-G24	4.0	0.19	2.6	0.11	6.6	0.25	At1g20620	Catalase 2
RAFL09-16-J23	2.0	0.37	5.2	0.08	5.3	0.02	At4g36040	DnaJ-like protein
RAFL11-11-J05	3.5	0.14	5.5	0.05	4.4	0.05	At5g19120	Conglutin gamma-like protein
Cellular communication/signal transduction								
RAFL02-02-B06	11.1	0.01	7.8	0.01	13.6	0.01	At2g33830	Putative auxin-regulated protein
RAFL02-03-L10	1.5	0.05	3.2	0.02	6.7	0.19	At3g49400	Transducin/WD-40 repeat protein family
RAFL02-06-N10	6.6	0.02	3.7	0.00	5.8	0.01	At5g14920	Proline-rich protein family
RAFL03-01-D10	2.1	0.08	6.8	0.06	14.0	0.13	At1g15670	Kelch repeat containing F-box protein family
RAFL03-06-N24	5.7	0.01	2.8	0.00	5.7	0.02	At5g14920	Proline-rich protein family
RAFL03-08-F09	1.6	0.19	2.0	0.09	5.7	0.15	At3g25770	Allene oxide cyclase family
RAFL04-13-H08	1.4	0.16	1.9	0.01	7.3	0.13	At5g18470	Putative receptor protein kinase
RAFL04-17-D16	9.2	0.04	3.7	0.15	6.5	0.03	At2g28840	RING zinc finger ankyrin protein related
RAFL04-20-D21	1.3	0.10	2.1	0.13	5.5	0.14	At2g13800	Receptor-like protein kinase
RAFL05-07-D07	3.7	0.05	2.4	0.00	7.0	0.03	At5g65930	Kinesin-like calmodulin-binding protein
RAFL05-10-H10	3.4	0.01	7.6	0.12	6.2	0.02	At3g62550	ER6 protein related
RAFL05-12-B21	1.9	0.04	1.6	0.57	5.9	0.30	At2g41100	Calmodulin-like protein
RAFL05-14-A14	1.7	0.04	1.5	0.05	5.9	0.14	At1g48210	Pto kinase interactor 1, putative
RAFL05-19-C01	1.3	0.19	1.7	0.01	7.3	0.24	At4g26070	MEK1
RAFL05-19-N20	2.1	0.05	4.2	0.04	6.0	0.06	At1g24530	Transducin/WD-40 repeat protein family
RAFL07-13-C20	1.8	0.05	2.8	0.05	6.1	0.04	At3g10300	Calcium-binding EF-hand family protein
RAFL09-10-B14	1.1	0.23	2.0	0.02	5.5	0.10	At5g39670	Calcium-binding, protein-like
RAFL09-14-D18	1.6	0.14	2.1	0.00	5.3	0.00	At5g35735	Auxin-induced protein family
Cellular organization ^c								
RAFL11-11-M23	1.8	0.02	3.6	0.03	7.0	0.01	At4g12470	pEARLI 1-like protein
Cellular transport and transport mechanisms								
RAFL08-13-H19	1.8	0.08	4.0	0.10	7.2	0.04	At3g52400	Syntaxin-like protein synt4
Energy								
RAFL04-10-L08	1.3	0.26	1.6	0.02	7.3	0.14	At4g34200	Phosphoglycerate dehydrogenase-like protein
RAFL04-20-I24	1.2	0.04	2.2	0.02	5.6	0.04	At2g44790	Phytocyanin, blue copper-binding protein II
Metabolism								
ERD11	1.7	0.13	1.7	0.40	22.2	0.22	At1g02930	Glutathione S-transferase
RAFL03-05-I07	2.0	0.15	1.3	0.20	16.3	0.09	At4g02520	Atpm24.1 glutathione S transferase
RAFL04-09-D07	1.4	0.16	2.3	0.03	6.9	0.21	At1g54100	Aldehyde dehydrogenase, putative
RAFL04-13-A13	1.4	0.14	3.6	0.07	6.5	0.15	At5g51830	Fructokinase 1
RAFL04-16-A17	2.0	0.07	3.5	0.07	5.7	0.05	At1g07240	UDP-glycosyltransferase family
RAFL04-16-P21	1.2	0.30	2.1	0.02	11.8	0.21	At4g37370	Cytochrome P450 - like protein
RAFL05-03-O21	1.2	0.22	1.9	0.01	6.5	0.10	At5g05600	Leucoanthocyanidin dioxygenase-like protein
RAFL05-13-F01	0.9	0.67	0.9	0.18	6.7	0.29	At1g74100	Putative flavonol sulfotransferase
RAFL05-18-E19	2.3	0.01	3.4	0.01	7.9	0.09	At1g08930	ERD6 protein
RAFL05-21-C06	1.2	0.61	2.4	0.09	18.3	0.33	At2g24850	Putative tyrosine aminotransferase

(continued on next page)

^a ([Fluorescence intensity (FI) of each cDNA for inoculated condition]/[FI of each cDNA for uninoculated condition])/median of ([FI obtained from the lambda control template DNA fragment for inoculated condition]/[FI obtained from the lambda control template DNA fragment for uninoculated condition]).

^b Protein entry code in the MIPS *Arabidopsis thaliana* Database (MAtdB) corresponding to the cDNAs is indicated.

^c Proteins are localized to the corresponding organelle.

Table 2. (continued from preceding page)

Clone name	Time after inoculation (h)						MIPS ^b	Annotation
	10		24		48			
	Ratio ^a	P value	Ratio	P value	Ratio	P value		
RAFL06-08-E09	1.1	0.24	1.5	0.04	13.1	0.08	At3g54640	Tryptophan synthase alpha chain
RAFL07-18-N04	1.6	0.21	3.6	0.03	13.0	0.23	At3g50740	UTP-glucose glucosyltransferase-like protein
RAFL09-09-L11	3.8	0.01	2.4	0.01	5.1	0.02	At3g14310	Pectin methylesterase-like protein
RAFL09-09-O05	2.0	0.02	1.7	0.02	7.5	0.10	At4g39980	2-dehydro-3-deoxyphosphoheptonate aldolase
RAFL09-11-D01	1.4	0.52	1.6	0.13	9.0	0.20	At1g74710	Isochorismate synthase 1 precursor (ICS1)
RAFL09-12-N24	2.5	0.01	2.0	0.09	9.0	0.05	At5g54810	Tryptophan synthase beta chain 1 precursor (sp P14671)
RAFL09-13-A04	3.0	0.01	2.2	0.02	11.9	0.10	At1g08920	Putative sugar transport protein
RAFL09-13-M13	2.9	0.00	5.8	0.17	5.8	0.14	At5g57560	TCH4 protein (gb AAA92363.1)
RAFL09-14-P21	1.8	0.02	2.2	0.03	5.4	0.13	At1g71100	Ribose 5-phosphate isomerase-like protein
RAFL09-15-O05	1.4	0.07	2.3	0.01	5.2	0.17	At3g19010	Oxidase-like protein
RAFL09-15-P17	5.2	0.08	3.2	0.12	5.1	0.15	At4g38550	Phospholipase-like protein
RAFL09-18-G13	1.2	0.12	2.0	0.02	5.3	0.16	At1g20510	Putative 4-coumarate:CoA ligase
Protein destination								
RAFL02-07-L08	1.7	0.04	3.1	0.06	16.2	0.17	At5g61160	Anthocyanin 5-aromatic acyltransferase-like protein
RAFL04-19-N11	1.5	0.04	8.1	0.04	10.8	0.10	At2g30610	POZ domain protein family
RAFL05-07-O03	1.6	0.05	3.1	0.05	5.2	0.08	At1g80920	Putative J8 protein
RAFL07-15-O03	2.2	0.01	3.9	0.04	29.1	0.18	At2g26560	Similar to latex allergen from <i>Hevea brasiliensis</i>
RAFL08-10-L17	1.4	0.08	2.0	0.04	7.5	0.09	At2g38860	(YLS5) proteaseI (pfpI)-like protein
RD19	6.4	0.01	3.1	0.00	5.1	0.00	At4g39090	Drought-inducible cysteine proteinase RD19A precursor
Transcription								
RAFL04-12-L23	4.3	0.02	6.5	0.03	9.5	0.02	At5g44260	Zinc finger-like protein
RAFL04-13-E17	1.0	0.23	2.8	0.06	21.7	0.09	At5g20230	Blue copper binding protein (bcb)
RAFL04-15-K19	2.0	0.02	7.8	0.24	14.8	0.16	At1g27730	Putative salt-tolerance zinc finger protein
RAFL04-16-M08	0.8	0.78	2.1	0.15	9.0	0.25	At3g56710	SigA binding protein
RAFL05-07-A18	3.0	0.08	3.0	0.03	7.1	0.05	At2g23320	Putative WRKY-type DNA-binding protein
RAFL05-19-O11	2.5	0.14	4.9	0.08	8.1	0.06	At4g29190	CCCCH-type zinc finger protein related
RAFL06-08-N24	1.5	0.02	5.0	0.14	6.7	0.10	At4g17490	Ethylene responsive element binding factor-like protein
RAFL06-09-A05	2.1	0.06	6.0	0.19	2.4	0.00	At5g51190	AP2 domain transcription factor, putative
RAFL06-09-D24	2.0	0.16	1.9	0.23	5.2	0.28	At4g14365	C3HC4-type zinc finger ankyrin repeat protein like
RAFL08-13-K22	3.4	0.03	6.8	0.05	6.2	0.04	At1g25560	AP2 domain transcription factor, putative
RAFL08-16-E19	1.8	0.01	3.8	0.05	5.5	0.10	At3g05200	RING-H2 zinc finger protein ATL6 (ATL6)
RAFL09-10-M16	2.0	0.02	3.5	0.04	6.5	0.09	At4g17500	Ethylene-responsive-element binding factor 1
RAFL09-16-K13	2.2	0.03	5.5	0.02	4.1	0.00	At1g68840	Putative DNA-binding protein RAV2
Transport facilitation								
RAFL04-15-G17	1.5	0.10	1.9	0.03	5.4	0.10	At2g38290	Putative ammonium transporter
RAFL05-01-M18	3.2	0.19	2.4	0.16	6.2	0.06	At4g13510	Ammonium transport protein (AMT1)
RAFL05-11-G05	1.8	0.01	6.1	0.20	7.1	0.15	At4g24570	Putative mitochondrial uncoupling protein
RAFL07-15-G18	1.5	0.07	1.9	0.07	6.4	0.12	At3g23560	Aberrant lateral root formation 5 (ALF5)
RAFL08-09-M05	0.8	0.78	1.3	0.34	5.3	0.19	At3g22600	Predicted GPI-anchored protein
RAFL08-12-A04	1.7	0.14	3.0	0.03	7.9	0.04	At1g08830	Superoxidase dismutase
RAFL08-12-P13	2.5	0.04	2.8	0.19	7.0	0.27	At1g59870	Putative ABC transporter
RAFL09-16-I02	1.3	0.22	3.2	0.03	7.0	0.15	At2g39210	Nodulin-like protein
Unclassified proteins								
RAFL02-03-F05	2.8	0.03	4.0	0.03	8.2	0.11	At1g69890	Unknown protein
RAFL02-06-A10	1.1	0.17	1.6	0.02	6.0	0.15	At2g18690	Unknown protein
RAFL02-10-H11	1.3	0.27	2.1	0.50	14.3	0.25	At3g50480	Unknown protein
RAFL03-02-F02	3.0	0.01	3.3	0.01	8.4	0.03	At5g64260	Phi-1-like protein
RAFL04-12-C05	1.6	0.06	2.3	0.09	5.8	0.18	At4g24360	Putative protein
RAFL04-12-C20	7.1	0.05	3.1	0.04	2.5	0.00	At4g04330	Hypothetical protein
RAFL05-01-A07	10.3	0.04	16.4	0.00	17.8	0.02	At3g15450	Unknown protein
RAFL05-01-D05	1.1	0.26	2.0	0.01	5.4	0.16	At4g39670	Unknown protein

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ments, whereas SA and *C. higginsianum* conditions resulted in less similar defense-induction profiles.

Identifying an *Arabidopsis* *R* gene locus associated with recognition of *C. higginsianum*.

F₁ progeny from a test cross between Eil-0 and Col-0 exhibited the same phenotype of resistance to *C. higginsianum* as the Eil-0 parent. F₂ progeny segregated 604:221 (3:1, $X^2 = 1.41$, $P = 0.24$) for resistance versus susceptibility to the pathogen (Table 3). Thus, Eil-0 appears to have a single dominant

allele that confers resistance to *C. higginsianum*. We named this *R* locus *RCH1* (for recognition of *C. higginsianum*). We mapped the *RCH1* locus by using polymerase chain reaction (PCR)-based microsatellite and amplified polymorphic sequence genetic markers. Our results indicated that the *RCH1* locus was linked to the simple sequence length polymorphism (SSLP) marker *nga8*, located at the chromosome 4. *RCH1* could be placed within the 14.7-centimorgan interval from *nga8* on the upper portion of *Arabidopsis* chromosome 4.

Table 2. (continued from preceding page)

Clone name	Time after inoculation (h)						MIPS ^b	Annotation
	10		24		48			
	Ratio ^a	<i>P</i> value	Ratio	<i>P</i> value	Ratio	<i>P</i> value		
RAFL05-01-M12	5.6	0.02	6.4	0.02	8.0	0.01	At1g28330	Dormancy-associated protein
RAFL05-02-P09	1.9	0.11	7.0	0.01	7.3	0.05	At4g27450	Unknown protein
RAFL05-05-N18	1.8	0.08	2.9	0.20	5.6	0.22	At5g18130	Unknown protein
RAFL05-07-D22	5.0	0.04	3.0	0.02	8.7	0.05	At2g01030	26S ribosomal RNA
RAFL05-09-D10	1.6	0.09	1.9	0.05	6.2	0.08	At1g03220	EDGP precursor
RAFL05-10-J18	4.0	0.00	7.8	0.18	7.2	0.06	At2g15890	Unknown protein
RAFL05-10-M08	1.4	0.14	2.2	0.01	6.1	0.17	At1g76600	Unknown protein
RAFL05-13-B18	3.8	0.03	3.2	0.02	5.3	0.00	At1g56220	Unknown protein
RAFL05-14-I02	1.6	0.08	2.9	0.01	5.9	0.06	At3g10020	Anchorage subunit of a-agglutinin; Aga1p
RAFL05-16-P22	6.2	0.02	2.9	0.01	5.7	0.02	At4g05150	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
RAFL05-17-A02	8.5	0.00	1.7	0.00	1.7	0.02	At3g20810	Unknown protein
RAFL05-19-E15	6.8	0.01	4.9	0.03	11.5	0.04	At2g15960	Unknown protein
RAFL06-08-K11	2.3	0.03	4.3	0.06	5.0	0.02	At2g27830	Unknown protein
RAFL06-09-E13	2.8	0.08	2.4	0.06	5.9	0.03	At2g01010	18S ribosomal RNA
RAFL06-11-I04	3.0	0.04	4.1	0.04	5.3	0.02	At4g35750	Unknown protein
RAFL06-13-H13	5.9	0.04	2.5	0.06	2.9	0.01	At4g26670	Unknown protein
RAFL06-15-H18	10.2	0.07	6.4	0.13	8.8	0.08	At3g26740	Light-regulated protein, putative
RAFL07-09-F22	4.1	0.07	6.9	0.00	6.6	0.04	At1g11260	Glucose transporter
RAFL07-11-C14	1.7	0.11	6.4	0.00	5.6	0.09	At4g27450	Unknown protein
RAFL07-16-B09	7.7	0.00	10.9	0.23	2.5	0.05	At3g48360	Unknown protein
RAFL08-09-P06	4.0	0.00	7.4	0.03	4.2	0.06	At5g22920	PGPD14 protein
RAFL08-17-G11	1.8	0.08	2.0	0.21	6.9	0.22	At4g33050	Putative protein
RAFL09-14-J11	1.7	0.01	4.9	0.02	10.7	0.16	At2g36220	Unknown protein
RAFL09-15-I08	2.0	0.01	3.6	0.01	7.6	0.09	At1g21670	Unknown protein
RAFL09-17-J19	1.9	0.16	1.6	0.16	5.6	0.18	At4g12720	Growth factor-like protein
RAFL11-02-G16	3.6	0.06	3.3	0.01	5.9	0.01	At1g25270	Unknown
RAFL11-13-H10	8.2	0.01	3.2	0.07	3.5	0.01	...	...

Table 3. Distribution of interaction phenotypes among F₁ and F₂ progeny of crosses between Col-0 and Eil-0 plants inoculated with *Colletotrichum higginsianum* (MAFF305635)

<i>Arabidopsis thaliana</i> cross (female × male)		F ₂ segregation analysis		Predicted ratio ^a (S:R)	χ^2	P
Ecotype	Generation	Susceptible	Resistant			
Col-0	Parent	46	0			
Eil-0	Parent	0	55			
Col-0 × Eil-0	F ₁	0	13			
Eil-0 × Col-0	F ₁	0	13			
Col-0 × Eil-0	F ₂	159	406	1:3	2.97	P = 0.08
Eil-0 × Col-0	F ₂	62	198	1:3	0.18	P = 0.65
	Total of F ₂	221	604	1:3	1.41	P = 0.24

^a For use in chi-squared test of selected models for predicting the number of host genes associated with incompatibility. Ratios are drawn from the observed behavior of selfed-progeny of F₁ progeny of cross between Col-0 and Eil-0.

Fig. 6. Hierarchical clustering of gene expression data in *Arabidopsis* Col-0 plant inoculated with *Colletotrichum higginsianum*. **A**, The 120 genes with expression ratios (*C. higginsianum*-infected/uninfected) of more than fivefold, at least at one time point, were regarded as *C. higginsianum* infection-inducible genes using a cDNA microarray. The hierarchical clustering of 120 genes was performed in response to the inoculation with *C. higginsianum* and treatments with 5 mM salicylic acid (SA), 100 μM methyl jasmonate (JA), and 1 mM ethephon (ET). **B**, The expansion figure of A. Each gene is represented by a single row of colored boxes, and a single column represents a time point. Induction (or repression) ranges from pale to saturated red (or green) with a fold-change scale bar. The scale bar shows the relationship between color intensity and log₂ ratio values. The lengths of the branches on the abscissa show the correlation between the treatments. The lengths of the branches on the ordinate indicate relationship among experiments across all of the genes included in the cluster analysis. Short branches indicate closer correlation than long branches.

DISCUSSION

The interaction between *C. higginsianum* and *A. thaliana* is an excellent model pathosystem for study of many aspects of the plant–fungal pathogen interaction, including plant defense responses, as well as the fungal infection process. *Colletotrichum* spp. display two main colonization strategies: subcuticular intramural colonization and intracellular colonization (Perfect et al. 1999). Species utilizing the former strategy include *C. capsici* on cowpea (*Vigna unguiculata*) (Bailey et al. 1992; Pring et al. 1995) and cotton (*Gossypium hirsutum* L.) (Roberts and Snow 1984), and *C. circinans* on onion (Walker 1921). The majority of *Colletotrichum* spp. display the latter colonization strategy, although the length of the biotrophic stage is variable, and may even be absent in some interactions (Perfect et al. 1999). Examples where distinct biotrophic stages have been observed by cytological means include the interactions between *C. lindemuthianum* and bean (Perfect et al. 1999), *C. truncatum* and pea (O’Connell et al. 1993), *C. destructivum* and cowpea (Latunde-Dada et al. 1996), *C. sublineolum* and sorghum (Wharton and Julian 1996), and *C. graminicola* and corn (Mims and Vaillancourt 2002). We must now add to this list the interaction between *C. higginsianum* and *Arabidopsis*. Our research confirms that host cells remain alive in a susceptible host during the earliest stages of the interaction (i.e. up until 2 to 3 dpi). An apparent callose deposit around one of the intercellular hyphae in Figure 2C (the upper one in this picture) is good evidence for at least a limited biotrophic stage, because the cell must be alive to deposit the callose around the hypha.

Formation of wall appositions, called papillae, is a common response of plant cells to wounding (Hombler and Foissner 1993) or fungal infection (Aist and Bushnell 1991; Smart 1991). The formation of papillae typically is rapid, taking place before the integrity of the host cells has been damaged. Papillae are formed in response to specific stimuli, including recognition of structural or chemical features of pathogens, or damage associated with invasion (Aist and Bushnell 1991; Smart 1991). Enhanced papillae formation is correlated with resistance of some barley cultivars to *Blumeria graminis* f. sp. *hordei* and is proposed to be the primary mechanism for this resistance (Huckelhoven et al. 1999). Papillae were produced at the site of *C. higginsianum* penetration during both compatible and incompatible interactions with *Arabidopsis*. Nonetheless, the fungus penetrated through the papillae into the host epidermal cell in all cases in both the Col-0 and Eil-0 plants. The similar results were obtained by Wharton and associates (1996) and by Mims and Vaillancourt (2002) in their EM studies of *Colletotrichum* spp. on sorghum and corn, respectively. This suggests that the papillae probably do not play an important role in resistance of these *Arabidopsis* ecotypes to *C. higginsianum*.

A rapid accumulation of ROS and hypersensitive cell death are correlated with disease resistance in many pathosystems (Greenberg 1997). There are some lines of evidence for a direct antimicrobial effect of ROS, as well as for an indirect role of ROS as messengers that activate defense response genes (Lamb and Dixon 1997). Additionally, massive ROS accumulation can trigger a localized hypersensitive cell death, which may result in the limitation of pathogen development (Levine et al. 1994). However, a direct causative role for hypersensitive cell death in resistance has not been proven, and there are examples in the literature that argue against such a role (Adam and Somerville 1996; Dinesh-Kumar et al. 2000; Slesinski and Ellingboe 1969).

We used 3,3-diaminobenzidine (DAB) and lactophenol-trypan blue staining to assess the accumulation of ROS and host

cell death in *Arabidopsis* leaves inoculated with *C. higginsianum*. In all of the highly or moderately resistant ecotypes, chlorotic and necrotic flecks reminiscent of hypersensitive cell death developed at sites of inoculation after 2 to 3 dpi. However, DAB staining revealed that the accumulation of ROS was not a rapid response to fungal invasion, occurring instead only after 3 dpi. It seems that plants accumulated low levels of ROS in cells surrounding sites of inoculation with *C. higginsianum* within 2 dpi, and so it could not be detected by DAB staining. Hypersensitive cell death seems to be responsible for resistance in these ecotypes to *C. higginsianum*. The production of ROS did appear to be correlated with the resistant response, because no ROS could be detected up to 4 dpi in the compatible interactions that were tested. Onset of hypersensitive cell death during putative biotrophic stage of the interaction also may play an important role in inhibition of fungal growth.

Phytoalexins are one important component of a general defense response in plants. In many cases, there is a good correlation between the degree of host resistance and the levels of phytoalexin accumulation (Conn et al. 1988; Hammerschmidt 1999; Long et al. 1985; Smith 1982). In several plant–pathogen interactions, phytoalexins are observed to accumulate around infection sites after inoculation with avirulent isolates of a pathogen but not after inoculation with virulent isolates (Darvill and Albersheim 1984; Essenberg et al. 1992; Hargreaves and Bailey 1978; Thomma et al. 1999b). There are also examples in which phytoalexin accumulation does not correlate with resistance. Rouxel and associates (1991) reported that the total accumulation of indole phytoalexins by various *Brassica* spp. could not be correlated with the resistance of each species to the fungal pathogen *Leptosphaeria maculans*. Pedras and Seguin-Swartz (1992) reported similar findings from their studies of the interactions between *L. maculans* and various cruciferous plants. In our study, the phytoalexin camalexin could scarcely be detected in the Eil-0 plants inoculated with *C. higginsianum* even 4 dpi. Expression of the *pad3* gene, which is associated with the synthesis of camalexin, was only slightly induced at 2 dpi. There are at least two possible explanations. First, camalexin may not play a role in the resistance of Eil-0 to *C. higginsianum*. A second possibility is that camalexin is important, but that the Eil-0 plants only accumulate high levels of the compound in the few cells immediately surrounding sites of inoculation with *C. higginsianum*, and so it could not be detected by our methods that involved extraction of larger quantities of tissue. Like Eil-0, susceptible Col-0 plants also did not produce detectable camalexin during early stages of infection with *C. higginsianum*, but accumulation was observed when necrotic, spreading lesions began to appear at 4 dpi. Transcripts of the *pad3* gene accumulated in inoculated Col-0 plants beginning at 2 dpi, and continued to increase in abundance through 4 dpi. The *pad3-1 Arabidopsis* mutant was increased markedly in its susceptibility to *C. higginsianum*. Thus, it is possible that camalexin does play a role in the defense of the Col-0 ecotype to *C. higginsianum*. To test whether camalexin plays a role in resistance to biotrophic fungal pathogens, the susceptibility of the different *pad* mutants was assessed to different strains of *Peronospora parasitica* and *Erysiphe orontii* (Glazebrook et al. 1997; Reuber et al. 1998). The resistance to these pathogens was not likely to be due to the deficiency in camalexin production. It also was investigated regarding the role of camalexin production in the resistance of *Arabidopsis* to the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea*. The results showed that the *pad3-1* mutant is markedly more susceptible than its wild-type Col-0 to infection with *A. brassicicola*, but not to *B. cinerea* (Thomma et al. 1999b). On the other hand, the *pad3* gene has functions in several pathways that are not yet entirely understood. Recently, expression profiling has shown that, in addition to a disrupted

camalexin biosynthesis, the *pad3-1* mutation alters the expression of numerous genes induced by *A. brassicicola* (Narusaka et al. 2003). Therefore, the camalexin cannot be the sole effector of *Arabidopsis* resistance.

Attempts by microbial pathogens to infect plants trigger activation of a defense signaling network. In *Arabidopsis*, there are three major defense-related pathways: these include the SA-mediated pathway, the JA-mediated pathway, and the ET-mediated pathway. The SA and JA pathways appear to be antagonistic (Rao et al. 2000; Seo et al. 1997; Shah et al. 1999) whereas the JA and ET pathways generally act synergistically (Penninckx et al. 1998). JA signaling is important for resistance to the necrotrophic fungal pathogen *A. brassicicola* (Thomma et al. 1998). ET and JA signaling both are important for resistance to another necrotrophic fungal pathogen, *B. cinerea* (Thomma et al. 1998; 1999a). A combination of SA-, JA-, and ET-dependent defenses is important for resistance to the bacterial pathogen *Xanthomonas campestris* pv. *armoraciae* (Ton et al. 2002). Generally, defense to biotrophic pathogens is dependent on SA, whereas defense responses to several necrotrophic pathogens have been shown to act through JA-, ET-, and ROS-dependent pathways (Delaney et al. 1994; Thomma et al. 1998; Tierens et al. 2002).

In our study, expression of the JA/ET-inducible genes *PR-4* and *PDFI.2* was induced in the resistant *Arabidopsis* Eil-0 plants and in the susceptible Col-0 plants after inoculation with *C. higginsianum*. In contrast, expression of the SA-dependent genes *PR-2* and *PR-5* did not appear to be strongly increased. Most of the 120 genes from our *Arabidopsis* cDNA microarrays that were induced by inoculation of Col-0 with *C. higginsianum* also were induced by treatment with JA or ET. This suggests that JA and ET signaling are more important than SA signaling in the defense response of *Arabidopsis* to *C. higginsianum*. However, the SA-dependent pathway does appear to play some role because there were a few genes that were induced only by infection with *C. higginsianum* or by treatment with SA. Patterns of defense responses induced by necrotrophic pathogen *Alternaria brassicicola* were found to be more similar to responses induced by ET treatment, but less similar to responses induced by SA (Huitema et al. 2003; Maleck et al. 2000; Schenk et al. 2000). Inoculation with *Peronospora parasitica* or *Pseudomonas syringae* (AvrRpt2)-resulted in defense-induction profiles similar to those induced by the SA analog benzothiadiazole (BTH) (Huitema et al. 2003). Our results suggest that resistance to the hemibiotrophic pathogen works more like resistance to a necrotrophic pathogen.

In the *Arabidopsis* ecotype Eil-0, resistance against *C. higginsianum* appears to be determined by a dominant allele at a locus we have named *RCH1*. We suggest that incompatibility in this case is determined by an interaction between the product of this *R* gene and the product of a corresponding hypothetical *avr* gene in the pathogen. The resistant *RCH1* allele represented in ecotype Eil-0 appears to occur infrequently, because it was identified only once among 37 ecotypes of *Arabidopsis thaliana* collected from diverse locations around the world (Table 1). One possible explanation is that *Arabidopsis* do not encounter this pathogen very often in nature; therefore, resistance alleles have not been selected for this pathogen. There are other cases in which resistance alleles seem to be rare in the population; for example, alleles for recognition of *A. candida* also appear to be relatively infrequent (Holub et al. 1995). In the future, it will be important to analyze more *C. higginsianum* isolates and more *Arabidopsis* ecotypes to identify additional resistance loci and alleles, if they exist. Although numerous loci for resistance to obligate biotrophic pathogens have been identified in *Arabidopsis* (Adam and Somerville 1996; Holub et al. 1995), ours is the first identification and characterization of a resistance locus for a hemibiotrophic *Colletotrichum* sp.

MATERIALS AND METHODS

Fungal culture.

Isolates of the pathogenic fungus *C. higginsianum* (MAFF305635, MAFF305968, and MAFF305970) were obtained from MAFF Genebank, Japan. The isolates used in this study all were genetically similar to one another, and could be differentiated from isolates of *C. gloeosporioides*, based on arbitrarily primed PCR with a primer recognizing a repetitive motif (CAG)₅ (Moriwaki et al. 1997). We purified the isolates from a single spore when we obtained them from the collection. Cultures of the isolate were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit) at 24°C in the dark. Conidia were obtained by gentle scraping of cultures incubated for 7 to 10 days and filtering through two layers of sterile cheesecloth.

Plant growth and inoculations.

Arabidopsis plants were grown in soil for 28 to 30 days in a growth chamber at 22°C under a cycle of 12 h of light and 12 h of dark. Plants were inoculated by spraying the leaves with a spore suspension (5×10^5 spores ml⁻¹ in distilled water) of *C. higginsianum*. Alternatively, between two and six 5- μ l drops of the spore suspension were placed on each leaf. Inoculated plants then were placed in a growth chamber at 22°C with a cycle of 12 h of light and 12 h of dark and maintained at 100% relative humidity. Control plants were treated only with distilled water.

All of the nonmutant *Arabidopsis* ecotypes (Table 1), with the exception of Aa-0, Cvi-0, Col-gl, and Di-G, were obtained from the SENDAI *Arabidopsis* Seed Stock Center (SASSC). Seed from Aa-0, Cvi-0, Col-gl, and Di-G were purchased from Lehle Seeds (Round Rock, TX, U.S.A.). The *pad3-1 Arabidopsis* mutant line was obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *eds5-1*, *eds16-1*, and *Col-nahG* plants were provided by M. C. Wildermuth and F. M. Ausubel (Massachusetts General Hospital, Boston). The *jar 1-1* mutant was provided by P. E. Staswick (University of Nebraska, Lincoln, U.S.A.). All of the mutants originally were derived from the Columbia ecotype.

Characterization of the disease reaction phenotypes.

DRS were assigned for each ecotype based on ratings of two different components of the disease reaction. These included the relative extent of pathogen growth within the leaf, as assessed by lactophenol-trypan blue staining, and the severity of chlorosis or necrosis. The DRS at 6 dpi was rated on a scale from 1 to 4, with a score of 0 indicating that no necrotic or chlorotic flecking was observed and no fungal growth was apparent (water controls always had scores of 0). A DRS of 1 was assigned when small chlorotic or necrotic flecks were formed sporadically in response to inoculation, and when fungal growth was very limited. Lesions on ecotypes with a DRS rating of 1 did not expand significantly, and fungal colonization did not increase very much, over the course of the experiment. A score of 2 indicated that chlorotic or necrotic lesions usually were formed at sites inoculated with the pathogen, but that the size of these lesions and the degree of fungal growth within the tissues at 6 dpi both were visibly reduced relative to the Col-0 ecotype. A score of 3 indicated the presence of brown necrotic lesions surrounded by a chlorotic halo, and dense fungal growth within the tissues, at 6 dpi. A score of 4 indicated rapid development of spreading necrotic lesions, compared with Col-0 plants, and the presence of wilted leaves (only the mutant *pad3-1* had a DRS this high).

Light and electron microscopy.

Between two and six 5- μ l drops of a spore suspension containing 5×10^5 spores ml⁻¹ in distilled water were placed on

Arabidopsis leaves. Fungal hyphae within resulting lesions were visualized by staining the inoculated leaves with lactophenol-trypan blue as described previously (Narusaka et al. 2003).

For detection of H₂O₂ in inoculated leaves, endogenous peroxidase-dependent *in situ* histochemical staining using DAB was performed as described previously (Narusaka et al. 2003).

For electron microscopy, *Arabidopsis* plants were inoculated with *C. higginsianum* by placing two 5- μ l drops of a suspension of 2×10^6 spores ml⁻¹ in distilled water on each leaf. The inoculated plants were kept at 22°C for between 1 and 6 days in a moist chamber. The inoculated leaves were pre-fixed with 2.5% glutaraldehyde in 67 mM phosphate buffer at pH 7.4 at room temperature overnight. After rinsing with the buffer, the leaves were post-fixed with 1% osmium tetroxide buffered with 67 mM phosphate buffer at pH 7.4 for 1 h at room temperature. The specimens were dehydrated with ethanol and embedded in Quetol 651 resin mixture or Spurr resin. Sections (90 nm) were cut with an ultramicrotome MT-1 by using a diamond knife, and then stained with 4% uranyl acetate for 10 min and lead citrate for 10 min. The stained sections were observed with an electron microscope (Hitachi 7100, Hitachi Corp., Hitachinaka-city, Japan).

Determination of camalexin levels.

Camalexin quantitation was done according to the procedure described by Thomma and associates (1999b).

RNA extraction and gel blot analysis.

Total RNA was isolated from the leaves that had been flash-frozen in liquid nitrogen immediately after harvesting. Total RNA was prepared using TRIZOL Reagent according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, U.S.A.). Messenger RNA (mRNA) was prepared using an mRNA isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). RNA gel blot hybridization analysis was carried out essentially as reported previously (Narusaka et al. 2003).

Microarray analysis.

Full-length cDNA microarray analysis was carried out essentially as reported previously (Narusaka et al. 2003; Seki et al. 2001, 2002). *Arabidopsis* Col-0 plants were grown in soil for 28 days in a growth chamber at 22°C under a cycle of 16 h of light and 8 h of dark. Plants were inoculated with *C. higginsianum* strain MAFF305635 by spraying them with a spore suspension (5×10^5 spores ml⁻¹ in distilled water). For other treatments, plants were sprayed with 5 mM SA; 100 μ M methyl jasmonate (JA); or 1 mM ethephon (ET). Control plants were sprayed with distilled water. All plants then were returned to the growth chamber and maintained at a high humidity.

Total RNA was isolated 10, 24, and 48 h after fungal inoculation, 2, 5, 10, and 24 h after SA, JA, or ET treatments, and 2, 5, 10, 24, and 48 h after spraying with distilled water. Leaves were harvested and then frozen immediately in liquid nitrogen until further analysis. In this study, we used an *Arabidopsis* microarray containing approximately 7K *Arabidopsis* full-length cDNAs and the drought-inducible genes *RD* and *ERD*. The cDNAs with expression ratios (treated/untreated) greater than fivefold that of the lambda control template DNA fragment (TX803; Takara, Kyoto, Japan) at one or more time points were considered to be pathogen- and signal molecule-inducible genes (Narusaka et al. 2003; Seki et al. 2001, 2002). Untreated Col-0 plants grown under the same conditions as the treated plants were used for calculations of fold induction for all treatments, including the water control.

Image analysis and signal quantification were performed with a QuantArray version 2.0 (GSI Lumonics, Oxnard, CA).

Background fluorescence was calculated on the basis of the fluorescence signal of two negative control genes (the mouse nicotinic acetylcholine receptor epsilon-subunit [nAChRE] gene and the mouse glucocorticoid receptor homolog gene). Genes showing a signal value <1,000 in both the Cy3 and the Cy5 channels were not considered. The median of 12 signal values obtained from the lambda control template DNA fragment was used as an external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with GeneSpring (Silicon Genetics, San Carlos, CA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

RIKEN Genomic Sciences Center *Arabidopsis* Genome Encyclopedia website: range.gsc.riken.go.jp