Characterization of the Early Response of Arabidopsis to *Alternaria brassicicola* Infection Using Expression Profiling

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All tested accessions of Arabidopsis are resistant to the fungal pathogen *Alternaria brassicicola*. Resistance is compromised by *pad3* or *coi1* mutations, suggesting that it requires the Arabidopsis phytoalexin camalexin and jasmonic acid (JA)-dependent signaling, respectively. This contrasts with most well-studied Arabidopsis pathogens, which are controlled by salicylic acid-dependent responses and do not benefit from absence of camalexin or JA. Here, mutants with defects in camalexin synthesis (*pad1, pad2, pad3*, and *pad5*) or in JA signaling (*pad1, coi1*) were found to be more susceptible than wild type. Mutants with defects in salicylic acid (*pad4* and *sid2*) or ethylene (*ein2*) signaling remained resistant. Plant responses to *A. brassicicola* were characterized using expression profiling. Plants showed dramatic gene expression changes within 12 h, persisting at 24 and 36 h. Wild-type and *pad3* plants responded similarly, suggesting that *pad3* does not have a major effect on signaling. The response of *coi1* plants was quite different. Of the 645 genes induced by *A. brassicicola* in wild-type and *pad3* plants, 265 required *COI1* for full expression. It is likely that some of the *COI1*-dependent genes are important for resistance to *A. brassicicola*. Responses to *A. brassicicola* were compared with responses to *Pseudomonas syringae* infection. Despite the fact that these pathogens are limited by different defense responses, approximately 50% of the induced genes were induced in response to both pathogens. Among these, requirements for *COI1* were consistent after infection by either pathogen, suggesting that the regulatory effect of *COI1* is similar regardless of the initial stimulus.

Plants respond to microbial pathogen attack by activating an array of inducible defense responses. Activation is controlled by a complex signal transduction network that has been studied using Arabidopsis genetics and genomics methods (Maleck et al., 2000; Schenk et al., 2000; for review, see Glazebrook, 2001; Glazebrook et al., 2003). Attack by some pathogens triggers accumulation of the signal molecule salicylic acid (SA), which in turn causes expression of several defense effector genes, such as *PR1* (*pathogenesis-related 1*), *BG2* (*beta-glucanase 2*), and *PR5* (*pathogenesis-related 5*; Ward et al., 1991). Pathogen attack may also trigger accumulation of the signal molecules jasmonic acid (JA) and ethylene (ET), which cause activation of a different set of defense effector genes, including plant defensin (*PDF1.2*) (Penninckx et al., 1998), a hevein-like protein, and a chitinase (Thomma et al., 1998). The SA- and JA/ET-mediated pathways interact in a complex fashion including mutual inhibition between JA/ET and SA signaling (for review, see Pieterse et al., 2001; Kunkel and Brooks, 2002; Glazebrook et al., 2003).

Numerous Arabidopsis mutations that affect SA signaling have been identified. Plants with *eds1* or *pad4* defects fail to activate SA accumulation after attack by certain pathogens (Zhou et al., 1998; Feys et al., 2001). Mutations in *SID2* likely block SA biosynthesis because *SID2* encodes an isochorismate synthase that appears to be required for SA synthesis (Wildermuth et al., 2001). Mutations in *EDS5*, which encodes a MATE class transporter, also result in extremely low SA levels, suggesting that *EDS5* is also required for SA synthesis (Nawrath and Metraux, 1999; Nawrath et al., 2002). Plants carrying the *NahG* transgene have very low SA levels because *NahG* encodes a salicylate hydroxylase that degrades SA as it is formed (Delaney et al., 1994). Mutations in *npr1* block many responses to SA (Cao et al., 1994). All of these mutants display enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* and the oomycete pathogen *Peroonospora parasitica*, indicating that defense responses under SA control are critical for resistance to these pathogens (for review, see Glazebrook, 2001).

In contrast, SA is not required for resistance to the fungal pathogen *Alternaria brassicicola* because *NahG* plants retain the strong resistance to this pathogen characteristic of the Arabidopsis accession Columbia (Col; Thomma et al., 1998). JA signaling seems to be
required instead because mutations in COII1 cause susceptibility to A. brassicicola (Thomma et al., 1998). COII encodes an F-box protein required for responses to JA (Xie et al., 1998; Xu et al., 2002). JA levels increase greatly in response to A. brassicicola infection, consistent with the idea that JA is an important signal molecule for A. brassicicola resistance (Penninckx et al., 1996). Mutations in PAD3 also cause susceptibility to A. brassicicola (Thomma et al., 1999b). PAD3 encodes the cytochrome P450 mono-oxygenase CYP71B15, and mutants with pad3 defects fail to produce camalexin after pathogen infection, presumably because CYP71B15 is an enzyme involved in camalexin biosynthesis (Glazebrook and Ausubel, 1994; Zhou et al., 1999). Therefore, resistance to A. brassicicola seems to require both camalexin synthesis and JA signaling. Application of JA does not induce camalexin accumulation, and coi1 mutants are not defective in camalexin synthesis after A. brassicicola infection, indicating that synthesis of camalexin is under control of a JA-independent pathway (Thomma et al., 1999b). JA treatment of pad3 mutants restores resistance to A. brassicicola (Thomma et al., 1998), indicating that activation of JA-mediated responses before infection is sufficient for resistance.

Relatively few genes required for JA signaling are known. In addition to COII1, JAR1 is required for some JA-dependent responses. JAR1 is involved in adenylation of JA (Staswick et al., 2002). Resistance to A. brassicicola is not affected by jar1 (S.C.M. van Wees and J. Glazebrook, unpublished data). Several Arabidopsis genes are known to be required for JA biosynthesis, including LOX2, DAD1, AOS, and OPR3 (Bell et al., 1995; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002; Von Malek et al., 2002). There are no reports on the effects of these mutations on A. brassicicola resistance.

Plant responses to A. brassicicola infection are not as well understood as those to other pathogens such as P. syringae and P. parasitica, which are controlled by SA signaling pathways. Schenk et al. (2000) reported a microarray study that identified 168 genes induced by A. brassicicola 3 d after infection. An Arabidopsis mutant, esa1, with enhanced susceptibility to A. brassicicola was identified, but the affected gene has not been isolated (T ierrens et al., 2002). The esa1 mutant shows delayed induction of PDF1.2 gene expression and camalexin accumulation upon challenge with A. brassicicola and is less responsive to reactive oxygen species (T ierrens et al., 2002). Studies of the A. brassicicola-Arabidopsis system have the potential to reveal the organization of the JA-dependent signal transduction circuitry and the nature of plant genes involved in resistance to fungal pathogens.

Here, a survey of A. brassicicola resistance in various Arabidopsis mutants is described. Mutants with defects in camalexin synthesis or in JA signaling were more susceptible than wild type. Mutants with defects in SA or ET signaling remained resistant. To characterize the response of Arabidopsis to A. brassicicola and identify genes that may play important roles in resistance, expression profiling of wild-type, pad3, and coi1 plants was carried out and compared with previous profiling of Arabidopsis infected with P. syringae (Glazebrook et al., 2003). Wild-type and pad3 plants responded similarly, exhibiting increased expression of 645 genes. Major gene expression changes occurred within 12 h of challenge, despite the fact that A. brassicicola had no visible effect on the plants, suggesting that defenses are activated rapidly in response to this pathogen. Among the 645 A. brassicicola-induced genes, 265 depended on COII1 for A. brassicicola-induced expression. Some of these genes may play important roles in resistance to A. brassicicola. Although P. syringae and A. brassicicola are limited by different defense responses, approximately 50% of the genes induced by one pathogen were also induced by the other. Among these, requirements for COII1 were consistent, suggesting that the regulatory effect of COII1 is similar regardless of the initial stimulus.

RESULTS

Resistance to A. brassicicola Is Compromised by pad1, pad2, pad3, pad5, and coi1 Mutations

To study the mechanisms underlying resistance to A. brassicicola, we surveyed Arabidopsis mutants with defects in production of camalexin (pad1, pad2, pad3, pad4, and pad5), JA signaling (coi1), ET signaling (ein2), and SA signaling (sid2). Figure 1A shows the disease symptoms 3 d after inoculation of leaves with A. brassicicola spores. Wild-type ecotype Col, ein2, sid2, and pad4 plants developed small, beige, necrotic lesions no larger than the initial inoculation droplet. In contrast, the other mutants exhibited spreading lesions. The lesions on pad1, pad2, and coi1 mutants were beige, whereas pad3 and pad5 developed brown lesions. Disease severity was quantified by classifying the infected leaves according to lesion diameter and color, as shown in Figure 1B.

Two methods were used to assess growth of A. brassicicola. In the first method, the number of spores produced at the infection site was determined. Figure 1C shows that few if any new spores were formed on A. brassicicola-inoculated wild-type, pad4, ein2, or sid2 plants, whereas new spores developed abundantly on all of the other genotypes. Using this spore count assay, enhanced susceptibility of a mutant might be missed if the mutant is not sufficiently susceptible to allow spore production. To address this problem, we determined fungal biomass using qPCR. In this assay, the amount of fungal biomass is measured as the qPCR signal from amplification of a fragment of fungal genomic DNA relative to the signal from amplification of a fragment of plant genomic DNA. Figure 1D shows that no fungal biomass was detectable in wild-type or pad4 plants, but fungal biomass was...
Figure 1. Quantification of disease severity in Arabidopsis mutants challenged with *A. brassicicola*. Measurements were taken 3 d after application of 5-µL droplets containing $5 \times 10^5$ spores mL$^{-1}$ on the sixth through 11th true leaves of 26-d-old plants. A, Lesion development. B, Distribution of disease severity classes. Disease severity is expressed as the percentage of leaves falling in disease severity classes: be, lesion is beige; br, lesion is brown; $<2$, $2<be/br<3$, $3$, $100\%$, lesion diameter is smaller than 2 mm, exactly 2 mm, between 2 and 3 mm, bigger than 3 mm, or expanding over the whole leaf, respectively. The inoculation droplet size is 2 mm. Data represent 40 to 60 leaves of 12 plants per genotype. Asterisks indicate statistically significant different frequency distributions of the disease severity classes compared with the wild type (Chi-square test; $\alpha = 0.05, n = 40–60$). C, Average number of in planta-formed spores per lesion $\pm$ SD. Each data point is the average of three pools of 18 inoculated leaves of four plants per genotype. Different letters indicate statistically significant differences between genotypes (Tukey’s honestly significant difference test on log-transformed data; $\alpha = 0.05, n = 3$). D, Growth of *A. brassicicola* in planta based on quantitative PCR (qPCR) with *A. brassicicola*- and Arabidopsis-specific primers/probes. Data points are the averages of the ratio of the qPCR signal of the *A. brassicicola*-specific gene cutinase (AbrCUT) and the Arabidopsis-specific gene *PR1* (*AthPR1*) of three replicates. DNA for the PCR reaction was isolated from circular tissue punches 6 mm in diameter centered on the lesion. Error bars $\pm$ SD. The experiments were repeated at least three times, and similar results were obtained.
measurable in the other pad mutants and in coi1, consistent with the results of the spore count assay. Consequently, it appears that when A. brassicicola is able to grow, it causes spreading lesions and produces spores. There were differences in the relative degree of susceptibility of the various mutants among the assays, but this probably reflects experimental variation rather than a real difference because there were similar differences among independent replicates of the same assay.

All of the measures of disease resistance we used show that pad1, pad2, pad3, pad5, and coi1 mutants are more susceptible to A. brassicicola infection, whereas pad4, ein2, and sid2 are not. Previous work showed that pad3 and coi1 mutants were more susceptible to A. brassicicola (Thomma et al., 1999a, 1999b). In contrast to our results, those workers found that pad1 was not more susceptible (Thomma et al., 1999b). It is possible that differences in infection conditions are responsible for this discrepancy.

A. brassicicola Susceptibility of pad Mutants Correlates with Reduced Levels of Camalexin and JA Responsiveness

The camalexin deficient phenotype of pad4 is dependent on the nature of the challenging pathogen. Camalexin levels in pad4 challenged with a virulent P. syringae strain are much lower than in wild-type plants, whereas after challenge with certain avirulent P. syringae strains or the fungus Cochliobolus carbonum, camalexin levels in pad4 plants are indistinguishable from wild type (Zhou et al., 1998). To test for similar phenomena, we assayed camalexin levels in wild-type and pad mutant plants after challenge by A. brassicicola. Figure 2A shows that camalexin levels in infected pad1, pad2, pad3, and pad5 plants were lower than in wild type. However, camalexin levels in pad4 plants were similar to wild type, indicating that PAD4 is not required for camalexin synthesis triggered by A. brassicicola infection. Camalexin levels in coi1 plants were also similar to wild type. The fact that all of the pad mutants displaying enhanced susceptibility to A. brassicicola also showed reduced camalexin levels provides additional support for the idea that camalexin is required for resistance to A. brassicicola. Because camalexin levels are not affected in coi1 plants, susceptibility of coi1 plants must be unequal variances; α = 0.05, n = 4). B, Expression of PDF1.2 36 h after challenge with A. brassicicola of 26-d-old plants. RNA was extracted from circular tissue punches 6 mm in diameter centered on the lesion. Each data point represents the mean of three replicate quantitative reverse transcriptase-PCR (qRT-PCR) assays. Error bars = SD, calculated as described in “Materials and Methods.” C, Expression of PDF1.2 12 h after treatment with MeJA. Plants were grown on soil for 14 d, then sprayed with 50 μM MeJA until runoff. Six seedlings were combined for each sample. Each data point represents the mean of three replicate qRT-PCR assays. Error bars = SD, calculated as described in “Materials and Methods.”
Expression of the plant defensin gene PDF1.2 is induced by *A. brassicicola* infection and requires JA and ET signaling (Penninckx et al., 1996, 1998). To test for defects in this signal transduction pathway among *pad* mutants, we determined PDF1.2 mRNA levels in *A. brassicicola*-infected plants by qRT-PCR. Figure 2B shows that *coi1* and *pad1*, but not the other *pad* mutants, failed to activate PDF1.2 expression in response to infection. To test for defects in JA signaling downstream from JA production, we determined PDF1.2 mRNA levels in plants treated with MeJA. Figure 2C shows that although the other *pad* mutants activated PDF1.2 expression in response to MeJA treatment, *pad1* and *coi1* plants did not, consistent with our previous report of a JA-signaling defect in *pad1* plants (Glazebrook et al., 2003). Taken together, these results strongly suggest that the susceptibility of *pad1* plants to *A. brassicicola* results from two factors, reduced camalexin production and a defect in jasmonate signaling, whereas the susceptibility of *pad2*, *pad3*, and *pad5* plants is probably due to reduced camalexin production.

**A. brassicicola-Induced Gene Expression Changes Are Very Similar in Wild-Type and *pad3* Plants**

To characterize the response of Arabidopsis to *A. brassicicola* infection, we carried out an expression profiling experiment using an Arabidopsis GeneChip array (Affymetrix, Santa Clara, CA) representing approximately 8,000 Arabidopsis genes. Wild-type, *pad3*, and *coi1* plants were infected with *A. brassicicola* or mock treated and sampled after 12, 24, and 36 h. At all these time points, there were no visible differences in symptoms among the three genotypes. At 12 h, no lesions were visible, whereas at 24 and 36 h, necrotic lesions the size of the initial inoculation droplet were evident. Total RNA prepared from these samples was hybridized to the arrays and expression values for each gene were obtained as described in “Materials and Methods.”

Figure 3A shows that many probe sets indicated strongly increased gene expression in wild-type plants 12 h after infection, whereas few probe sets
indicated strongly repressed expression. Figure 3B shows that the gene expression patterns in infected wild-type plants were extremely similar to those in pad3 plants, whereas Figure 3C shows that in coi1 plants, many probe sets indicated altered expression. Similar patterns were observed at the 24- and 36-h time points.

We explored the extent of the similarity between wild-type and pad3 plants in the following way: Ratios of expression level in infected plants to expression level in mock-treated plants were calculated for wild-type and pad3 plants at all three time points. Probe sets that at any time point showed an increase of 2-fold or more in response to infection in wild-type or pad3 plants, and had an expression level of at least 1.0 in the A. brassicicola-infected sample, were selected. For this list of 1,490 probe sets (see Supplementary Table I at http://www.plantphysiol.org), the fold-change ratios were log10 transformed, and the uncentered Pearson correlation coefficients were calculated for each pair of samples, as shown in Table I. From this analysis, it is evident that the genes induced in response to infection in wild-type and pad3 plants are very similar, whereas there is more deviation between wild-type and coi1 plants. This leads to two conclusions. First, it shows that at 12 h after infection, when A. brassicicola has no visible effect on wild-type plants, the plants respond by activating a large number of genes. Second, it suggests that pad3 does not have a major effect on gene expression, consistent with the idea that the function of the putative cytochrome P450 monooxygenase encoded by PAD3 is camalexin biosynthesis.

**Definition of A. brassicicola-Induced Genes**

To reduce the number of false positives among genes judged to be induced by A. brassicicola, the data sets from wild-type and pad3 plants were treated as replicates. Two different methods were used to select induced genes, and the genes that were selected by both methods were considered to be A. brassicicola induced. In the first method, probe sets were required to have a signal of at least 1.0 in infected samples and to show at least a 2-fold increase in infected samples relative to the corresponding mock-treated samples, in both wild-type and pad3 plants, at any time point. Data from 948 probe sets met these conditions. Among these, 645 probe sets met the conditions at two or more time points, and 417 probe sets met them at all three time points, indicating that the expression patterns at the three time points are quite similar. In the second method, the Welch t test module of GeneSpring was used to select significantly induced genes at each time point, treating wild-type and pad3 samples as replicates. The Welch t test was applied to the probe sets that had a signal of at least 1.0 in infected samples and that showed a fold change greater than 1.001 when comparing infected samples with the corresponding mock-treated samples. Thus, probe sets showing a fold change of less than 2 can pass the test as long as they show a statistically significant increase in expression level after infection. Data from 975 probe sets passed the test for at least one time point. Among these, 353 passed for at least two time points, and 95 passed for all three time points. Of the 948 probe sets that passed the 2-fold change test, 728 (77%) also passed the Welch t test for at least one time point. Because these 728 probe sets passed both tests, it is likely that the 645 genes represented by these probe sets are significantly induced by A. brassicicola infection. They are shown in red in Figure 4A and listed in Supplementary Table II (see http://www.plantphysiol.org).

**Many A. brassicicola-Induced Genes Require COI1 for Expression**

The fact that the JA-signaling mutant coi1 is susceptible to A. brassicicola suggests that at least one A. brassicicola-induced gene whose expression is reduced in coi1 plants is important for resistance. COI1-dependent genes were identified among the list of 728 probe sets representing A. brassicicola-induced genes in the following way: The signal ratios for infected coi1-infected wild type and infected coi1-infected pad3 were calculated for each time point. Probe sets for which both ratios were less than 0.5 (representing a 2-fold reduction in coi1 relative to wild type and pad3) for at least one time point were considered to represent COI1-dependent genes. There were 293 such probe sets, representing 265 genes (see

<table>
<thead>
<tr>
<th>Table I. Pearson correlation coefficients calculated over 1,490 probe sets</th>
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<tr>
<td>The uncentered Pearson correlation coefficients for the indicated pairs of log10-transformed ratios are shown.</td>
</tr>
<tr>
<td>Col Alt/mock 12</td>
</tr>
<tr>
<td>Col Alt/mock 24</td>
</tr>
<tr>
<td>Col Alt/mock 36</td>
</tr>
<tr>
<td>pad3 Alt/mock 12</td>
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<tr>
<td>pad3 Alt/mock 24</td>
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<tr>
<td>pad3 Alt/mock 36</td>
</tr>
<tr>
<td>coi1 Alt/mock 12</td>
</tr>
<tr>
<td>coi1 Alt/mock 24</td>
</tr>
<tr>
<td>coi1 Alt/mock 36</td>
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</table>
Figure 4. Scatter plot comparison of A. brassicicola-induced genes. The diagonal lines define 2-fold changes. Both axes are logarithmic. A, For all probe sets, the normalized expression value for wild-type plants infected with A. brassicicola and sampled at 12 h hpi is shown on the vertical axis, and the value for mock-treated wild-type plants is shown on the horizontal axis. Probe sets representing A. brassicicola-induced genes are shown as red dots. B, The normalized expression value in coi1 plants infected with A. brassicicola and sampled at 12 h is shown on the vertical axis, and the corresponding value for wild-type plants is shown on the horizontal axis. Only probe sets representing A. brassicicola-induced genes are shown. Probe sets representing COI1-dependent genes are shown as red dots.

Supplemental Table III at http://www.plantphysiol.org. Among these, 155 probe sets met the condition for at least two time points, and 82 met the condition for all three time points. Figure 4B shows a comparison of probe sets representing genes induced by A. brassicicola in wild type compared with coi1 at the 12-h time point. The 293 probe sets from the COI1-dependent list are shown in red. Of these probe sets, 80% showed at least a 2-fold reduction relative to wild type at the 12-h time point. Note that this list of COI1-dependent A. brassicicola-induced genes is less reliable than the list of A. brassicicola-induced genes because it is based on just one set of coi1 data using a 2-fold change criterion, and statistical tests were not possible.

The list of COI1-dependent A. brassicicola-induced genes includes genes known to be JA induced. Table II shows that expression levels of the JA-regulated effector genes PDF1.2, VSP, and HEL were greatly reduced in the coi1 mutant. Strikingly, induced expression of the JA biosynthetic genes LOX2 and AOS was completely dependent on coi1. Although it was known that these genes are induced by JA, our results show that their induction after A. brassicicola infection requires COI1, indicating the presence of a feed-forward loop that requires COI1. Curiously, induced expression of OPR3, which is required at a later step in JA biosynthesis, did not appear to be dependent on COI1, suggesting the existence of an additional regulatory component affecting expression of this gene.

Treeview Representation of Induced Genes

To create a visual representation of the data, the 728 A. brassicicola-induced probe sets were sorted according to similar expression patterns using Cluster (Eisen et al., 1998). The ratios of wild type infected/mock, pad3 infected/mock, coi1 infected/wild-type infected, and coi1 infected/pad3 infected, at all three time points, were clustered according to similar effects on gene expression. Figure 5 shows that the most closely related data sets are wild type and pad3 sampled at the same time point, and that A. brassicicola-induced genes tend to be induced at all three time points, in both wild-type and pad3 plants.

Table II. Expression levels of JA-regulated genes

<table>
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<tr>
<th>Gene Name</th>
<th>Probe Set Identification</th>
<th>Arabidopsis Genome Initiative Identification</th>
<th>Col 12</th>
<th>pad3 12</th>
<th>coi1 12</th>
<th>Col 24</th>
<th>pad3 24</th>
<th>coi 1 24</th>
<th>Col 36</th>
<th>pad3 36</th>
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<td>201/1.9</td>
<td>229/8.2</td>
<td>0.8/0.1</td>
<td>157.6/0.3</td>
<td>196.4/0.1</td>
<td>0.8/0.2</td>
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<td>0.04/0.0</td>
<td>77.5/2.0</td>
<td>33.0/0.7</td>
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<td>34.3/0.8</td>
<td>0.5/0.1</td>
<td>29.3/0.4</td>
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<td>0.08/0.1</td>
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<tr>
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<td>271/82.2</td>
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<td>36.6/21.3</td>
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<td>23.8/2.8</td>
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<td>25.1/2.9</td>
<td>34.3/2.3</td>
<td>16.7/2.6</td>
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</table>
Most of the genes that require COI1 for expression are expressed at reduced levels in coi1 plants at all time points. An expanded version of Figure 5 showing the dendrogram for the probe sets and labeled with gene names and files suitable for viewing in Treeview are provided as Supplemental Material (see www.plantphysiol.org).

**Relationship to P. syringae-Induced Genes**

Previously, we reported an expression profiling study of responses to *P. syringae pv maculicola* (Glazebrook et al., 2003). In that work, signal strength was calculated using an older version of the Affymetrix MAS software, and induced genes were defined based solely on fold-change ratios. For comparison with *A. brassicicola*-induced genes, we recalculated the signal using the method used for the *A. brassicicola* data (see “Materials and Methods”) for the three experiments that included wild-type plants 30 h after infection with *P. syringae* or mock treatment and for the one experiment that used coi1 plants 30 h after infection with *P. syringae*. Then, probe sets were selected that met the conditions of: (a) a signal of at least 1.0 in the infected samples, (b) signal in infected samples 2-fold higher than the signal in corresponding mock samples, and (c) conditions (a) and (b) were met in at least two of the three replicate experiments. There were 1,352 such probe sets. Next, the Welch t test was used to select probe sets that were significantly induced by infection using the three replicate data sets. There were 1,392 such probe sets. The overlap between the two lists was 1,100 probe sets, representing 976 genes. These probe sets were considered to have a high probability of representing *P. syringae*-induced genes and were used for further analysis. The normalized data for these genes are provided in Supplemental Table IV (see http://www.plantphysiol.org).

The probe sets on the *A. brassicicola*-induced list (728) were compared with those on the *P. syringae*-induced list (1,100). Four hundred seventy-four probe sets were common to both lists. Thus, despite the fact that resistance to *P. syringae* and *A. brassicicola* require quite different signaling processes, there are many genes that are induced by both pathogens. Among the 474 probe sets common to both lists, 193 were COI1 dependent after *A. brassicicola* infection, and 196 probe sets showed a signal that was at least 2-fold lower in *P. syringae*-infected coi1 plants than in *P. syringae*-infected wild-type plants from the same experiment. Between these two lists of probe sets representing COI1-dependent genes, 141 were in the log10 ratio for each probe set in each data set is indicated by a colored line. Increasing intensity of red indicates increasing values above 0, and increasing intensity of green indicates decreasing values less than 0. See Supplemental Materials (www.plantphysiol.org) for an expanded version of this figure.

**Figure 5.** Treeview representation of clustering results using data for the 728 *A. brassicicola*-induced probe sets. The statistic used for clustering was the log10 of the ratios indicated at the top of the figure. Probe sets are arranged in the vertical dimension, and the value of
common. Figure 6A shows a scatter plot of the expression levels of the probe sets in the *P. syringae*-induced, *COI1*-dependent list (397 probe sets) in wild type and *coi1* 12 h after *A. brassicicola* challenge. Figure 6B shows a scatter plot comparison of the expression levels of the probe sets in the *A. brassicicola*-induced, *COI1*-dependent list (293 probe sets) in wild-type and *coi1* plants 30 h after infection with *P. syringae*. In Figure 6, A and B, probe sets that are in both the *P. syringae*-induced, *COI1*-dependent and *A. brassicicola*-induced, *COI1*-dependent lists are indicated by red dots. For 90% of the probe sets in these two lists, the signal is lower in *coi1* than in wild type after infection by the other pathogen, but only 36% and 48% (indicated in red), respectively, pass the restrictions that we assigned to both *A. brassicicola*- and *P. syringae*-induced, *COI1*-dependent probe sets. From this analysis, it is evident that even though infection by *A. brassicicola* and infection by *P. syringae* are quite different stimuli, genes under *COI1* control in one case are generally also under *COI1* control in the other case.

### DISCUSSION

Four different assays were used to assess *A. brassicicola* susceptibility: visual inspection, measurement of lesion diameter, spore counting, and biomass measurement by qPCR. All of these measures yielded similar results with respect to detection of mutants susceptible to *A. brassicicola*. In general, it is probably most efficient to obtain quantitative measures of susceptibility using the spore count assay because it is the most time and cost effective.

For the *pad* mutants, there was a perfect correlation between camalexin deficiency after *A. brassicicola* infection and susceptibility, strengthening the conclusion that camalexin is an important factor in resistance to *A. brassicicola*. Furthermore, the strong similarities between expression profiles of wild-type and *pad3* plants suggest that *pad3* does not affect activation of defense responses other than camalexin synthesis. In contrast to our observation that *pad1* is camalexin deficient and susceptible to *A. brassicicola*, Thomma et al. (1999b) found that it was not camalexin deficient and was resistant to *A. brassicicola*. This discrepancy may be due to differences in infection conditions. Nevertheless, the correlation between camalexin deficiency and susceptibility is maintained.

There is also a defect in JA signaling in *pad1* plants because they fail to express PDF1.2 in response to either *A. brassicicola* infection or JA treatment (Fig. 2; Glazebrook et al., 2003). Because JA signaling is important for *A. brassicicola* resistance, there are likely two factors contributing to susceptibility of *pad1* plants, possibly explaining the complete destruction of the plants within 1 week after infection, a level of susceptibility not observed in any other mutant. The effect of *pad1* on JA signaling is different from that of *coi1*. In response to *A. brassicicola* infection, there is very little increase in expression of *PR1* in wild type or any of the *pad* mutants, but it is strongly induced in *coi1* plants, consistent with the idea that *PR1* expression is repressed by JA signaling in a *COI1*-dependent manner (S.C.M. van Wees and J. Glazebrook, unpublished data). Unlike *coi1*, the *pad1* mutation does not relieve this repression, suggesting that its effect on JA signaling is either less severe or occurs at a different point in the pathway.

No defect in JA signaling was detected in *pad2*; therefore, the susceptibility of this mutant may be due to camalexin deficiency. However, *pad2* likely affects expression of other defense responses because it interferes with expression of many genes normally induced by *P. syringae* infection (Glazebrook et al., 2003). Therefore, it is likely that it affects other defense responses important for *A. brassicicola* resis-
tance in addition to camalexin biosynthesis. PAD5 has not been isolated, and relatively little character-
ization of pad5 has been done, so it is not known whether pad5 affects signaling or not.

Although pad4 mutants are camalexin deficient af-
ter infection by virulent *P. syringae* strains, they were not camalexin deficient after *A. brassicicola* infection. This result is not entirely unexpected because pad4 mutants are not camalexin deficient after infection by certain avirulent *P. syringae* strains or after infection by the non-host fungal pathogen *C. carbonum* (Glaze-
brook et al., 1997). Apparently, requirements for PAD4 can be bypassed by activation of an unknown, parallel signaling process (Zhou et al., 1998).

Figure 3A may suggest that many more genes are induced by *A. brassicicola* infection than are re-
pressed. On closer inspection, it is apparent that there are also many repressed genes but that the extent of the repression is modest. The repressed genes were studied in a manner similar to the one used for the induced genes. There was much less consist-
ency in the data for the repressed genes. There was less overlap between the sets of repressed genes in wild type and in pad3 and also between genes repressed at different time points. For these reasons, lists of repressed genes are less reliable than the lists of induced genes, and more replicate experiments would be needed to make a reliable list. For this reason, the repressed genes were not studied further.

In the initial planning of the expression profiling experiment, pad3 was included because it seemed likely that wild-type plants might not show much response to *A. brassicicola* because the fungus did not grow or cause any tissue damage beyond the site of infection. In marked contrast to this expectation, wild-type plants exhibited a very strong response that was not detectably slower or weaker than the response of pad3 plants, which support extensive fun-
gal growth and suffer extensive tissue damage at time points later than those studied by expression profiling. Clearly, wild-type plants detect the presence of the fungus and mount a rapid response.

Data from this Arabidopsis Affymetrix GeneChip have been proven to be highly reproducible when a single RNA sample is used for the comparisons (Zhu and Wang, 2000). However, when defining lists of genes that show altered expression under certain conditions, it is also necessary to consider effects of biological variation. Unfortunately, we did not have data from biological replicates available to us. In light of the strong similarity between the responses of wild-type and pad3 plants, we decided to treat these samples as replicates. This allowed us to apply sta-
tistical criteria to the selection of *A. brassicicola*-induced genes, greatly strengthening conclusions that particular genes are induced by *A. brassicicola* infection. There was also considerable similarity among lists of genes induced at the three different time points, providing further evidence that genes on the list are truly *A. brassicicola* induced.

There are several types of errors that could have resulted from the way we analyzed the data: (a) Any genes that are *A. brassicicola* induced in wild-type but not in pad3 plants were excluded from our list of *A. brassicicola*-induced genes. There remains a for-
mal possibility that there are a few genes directly or indirectly regulated by pad3. (b) Any genes that are *A. brassicicola*-induced in pad3 but not in wild-type plants were excluded. A response to active fungal growth might cause this expression pattern. Inspection of Figure 5 suggests that some genes are ex-
pressed differently in pad3 and wild-type plants, particularly at 24 and 36 h, and these could be very interesting. (c) There may be false positives in our list of *A. brassicicola*-induced genes. Some environ-
mental occurrence during the single experiment might have caused some genes to show increased expression in infected plants relative to uninfected plants.

Resistance to *A. brassicicola* is known to require COI1, and 265 of the *A. brassicicola*-induced genes required COI1 for induced expression. Consequently, it seems likely that some of these genes are important for resistance. It is also likely that some of the COI1-
dependent *A. brassicicola*-induced genes may be im-
portant for resistance. Among the genes that were induced by both *A. brassicicola* infection and *P. syrin-
gae* infection, most genes whose expression was COI1 dependent after infection with one pathogen were also COI1 dependent after infection with the other. This suggests that the regulatory mechanisms control-
ing defense responses act similarly in response to different pathogens and that the structure of the net-
work topology will be similar for each pathogen studied.

Although it would of course be possible to discuss the putative biochemical functions of the *A. brassici-
cola*-induced genes in the context of their possible roles in resistance to *A. brassicicola*, we think that there is little value in such discussions; therefore, we have chosen not to supply one. As an alternative, we suggest that an effective way to discover genes required for resistance to *A. brassicicola* will be to apply the powerful reverse genetics systems of Ara-
bidopsis to test mutants with defects in *A. brassicico-
la*-induced genes for susceptibility to *A. brassici-
cola*. We have found this approach to be very effective for identifying Arabidopsis genes that are im-
portant for limiting growth of *P. syringae* (J. Clarke and J. Glazebrook, unpublished data). Mutations that cause susceptibility and affect a gene likely to be involved in signaling could then be subjected to further expression profiling studies to eventually elucidate the topology of the signal transduction network responsible for activating *A. brassicicola* resistance mechanisms.
MATERIALS AND METHODS

Plant Genotypes and Growth Conditions

All mutants were derived from the Col accession. *coi1* was *coi1-1* (Feyes et al., 1994; Xie et al., 1998), *ein2* was *ein2-1* (Guzman and Ecker, 1990; Alonso et al., 1999), *npr1* was *npr1-1* (Cao et al., 1994, 1997), *sid2* was *sid2-5* (van Wees and Glazebrook, 2003), *pad1* and *pad2* were described by Glazebrook and Ausubel (1994), *pad3* was *pad3-1* (Glazebrook and Ausubel, 1994; Zhou et al., 1999), *pad4* was *pad4-1* (Glazebrook et al., 1996; Jirage et al., 1999), and *pad5* was *pad5-1* (Glazebrook et al., 1997). Mutants homozygous for *coi1* are male sterile and, therefore, cannot self-fertilize. To select homozygous *coi1/coi1* segregants from the progeny of *coi1/Col* heterozygous plants, seeds were germinated on 0.5× Murashige and Skoog medium (Sigma, St. Louis) supplemented with 50 μM MeJA. Wild-type and heterozygous plants developed short roots and purple leaves, whereas homozygous mutant plants developed normally (Xie et al., 1998) and were transplanted into soil when 10 d old. Plants were grown in soil in a controlled-environment chamber at 22°C and 80% relative humidity (RH) on a 12-h-light/12-h-dark cycle, with 125 μm m⁻² s⁻¹ cool-white fluorescent illumination.

**Alternaria brassicicola Infections**

*A. brassicicola* strain MUC1 20297 was obtained from Willem Broekart (Katholieke Universiteit Leuven, Belgium). It was cultured on 0.5× potato dextrose agar (Sigma) medium at 22°C with 125 μm m² s⁻¹ cool-white fluorescent illumination on a 12-h-light/12-h-dark cycle for 9 d. Subsequently, the spores were washed from the surfaces of the plate with water. Concentration of spores was determined using a hemacytometer and adjusted to 5 × 10⁶ spores mL⁻¹. Plants were inoculated by placing one or two droplets of 5 μL of suspension on to the surface of the sixth through 11th true leaves. For mock treatment, 5 μL droplets of water were placed onto the leaves. Inoculated plants were kept at 100% RH at 24°C for 12-h-light/12-h-dark cycle, with 125 μm m² s⁻¹ cool-white fluorescent illumination.

**Spore Count Assay**

Batches of infected leaves containing 18 lesions were excised and shaken vigorously in a test tube containing 0.01% (v/v) Tween 20. Leaves were removed, and the remaining suspension was centrifuged (200g for 10 min), and the spores were resuspended in 600 μL of water. A hemacytometer was used to count the spores. Spores formed in plants were distinguishable from spores used for the inoculation because they were colorless in contrast to the brown appearance of the inoculated spores.

**qPCR Assay for Determination of Fungal Biomass**

DNA levels of the *A. brassicicola*-specific gene *cutinase* (*AbrCUT*) relative to the DNA levels of the Arabidopsis-specific gene PDF1.2 were determined by qPCR using Taq-Man chemistry on an Applied Biosystems 7500 machine (Applied Biosystems, Foster City, CA). Reactions were performed according to the manufacturer’s instructions. *AbrCUT* primers and 6-carboxy-fluorescein 5’ end-labeled probes (Sigma Genosys, The Woodlands, TX) were as follows: primers, 5’-CAGCGTCCCAATGATGAC-3’ and 5’-GTAGGCCAAAACAGAACACC-3’; and probe, 5’-CCATACCAGCCTTCGAGGCCC-3’. *PR1* primers and probe were as described previously (Jirage et al., 2001). Three replicates of the qPCR assay were used for each sample. The combined error of the measurement of *AbrCUT* and *PR1* was calculated as: 

\[ \text{value cutoff} = \frac{\text{mean AbrCUT} - \text{mean PR1}}{\text{mean AbrCUT} + \text{mean PR1}} \times (\text{mean AbrCUT} - \text{mean PR1}) \]

**Camalexin Assay**

Camalexin production was assayed 36 h after *A. brassicicola* challenge. Leaf discs, 6 mm in diameter centered on the lesion, were excised from infected leaves. Each sample consisted of 18 such discs from three plants, and there were four samples per treatment. Camalexin was determined as described by Glazebrook and Ausubel (1994).

**MeJA Treatment and qRT-PCR**

Seedlings were grown on soil for 14 d. The evening before treatment, plants were placed under 100% RH. MeJA obtained (Aldrich, Milwaukee, WI) was used to prepare a solution of 50 μM MeJA, 0.1% (v/v) ethanol, and 0.02% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX). Seedlings were sprayed with this solution until runoff. After treatment, plants were kept at 100% RH.

**PDF1.2**

mRNA levels were determined by qRT-PCR using Taq-Man chemistry and normalized to TRX3 mRNA as described previously (Jirage et al., 2001). The probe used for TRX3 was 5’-AGACCTCACAGCAATGCTGCCAC-3’ and the primers were 5’-AACGGAAATCCAAGAACTGATTGTC-3’ and 5’- AGACCGGTGCAATGAACG-3’. Three replicates of the qRT-PCR assay were used for each sample. The combined error of the measurement of PDF1.2 and the measurement of the TRX3 normalization control was calculated as: 

\[ \text{value cutoff} = \frac{\text{mean PDF1.2} + \text{mean TRX3}}{\text{mean PDF1.2} + \text{mean TRX3}} \]

**Sample Preparation for GeneChip Experiment**

Wild-type Col, *pad3*, and *coi1* plants were 26 d old when inoculated with *A. brassicicola* or mock treated, as described above. RNA was isolated from 6-mm-diameter leaf discs centered on the inoculation droplet. The different genotypes were monitored for susceptibility to *A. brassicicola* using all four of the assays described in Figure 1, and the results were similar to those shown in Figure 1. RNA preparation, labeling, hybridization, and scanning were carried out as described previously (Zhu and Wang, 2000).

**Computational Analysis of GeneChip Expression Data**

Expression values were the signals from each probe set obtained using Affymetrix MAS 5.0 software, with scaling set to 100. The data were then imported into GeneSpring software, and the data from each array were normalized by median centering to a value of 1.0. Many of the probe sets on the array yielded very low values, causing the corresponding genes to be called “absent” by the MAS 5.0 software. Consequently, most probe sets that represent expressed genes have normalized expression values of 1.0 or greater. The ratio of expression values for each probe set of a particular sample and its corresponding control sample (infected/mock or *coi1* infected/wild type or *pad3* infected) was calculated. Only probe sets of which the larger expression value was greater than 1.0 were selected, thereby excluding the probe sets with very low expression values. The cutoff value of 1.0 was chosen because in scatter plot comparisons of closely related data sets, the scatter increased markedly at values below 1.0, suggesting that these values were strongly affected by noise. For Welch *t* test analysis of *A. brassicicola*-induced genes, only probe sets for which expression values in the infected sample was at least 1.001-fold greater than in the corresponding mock sample were selected. The Welch *t* test algorithm (Welch, 1947) of GeneSpring was used on log₂-transformed expression data to select significantly induced genes at each time point, treating wild-type and *pad3* samples as replicates and the treatment as the parameter for comparison. The Welch *t* test used is a parametric test that assumes that the variances are not equal. The P value cutoff was set at 0.05, and no multiple testing correction was applied. The uncentered Pearson correlation coefficients R (i.e. normalized dot products) were calculated as: 

\[ R = \frac{(A - \bar{A}) \cdot (B - \bar{B})}{\sqrt{(A - \bar{A}) \cdot (A - \bar{A}) \cdot (B - \bar{B}) \cdot (B - \bar{B})}} \]

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LITERATURE CITED


