Loss-of-Function Mutations in Chitin Responsive Genes Show Increased Susceptibility to the Powdery Mildew Pathogen Erysiphe cichoracearum

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Chitin is a major component of fungal walls and insect exoskeletons. Plants produce chitinases upon pathogen attack and chito-oligomers induce defense responses in plants, though the exact mechanism behind this response is unknown. Using the ATH1 Affymetrix microarrays consisting of about 23,000 genes, we examined the response of Arabidopsis (Arabidopsis thaliana) seedlings to chito-octamers and hydrolyzed chitin after 30 min of treatment. The expression patterns elicited by the chito-octamer and hydrolyzed chitin were similar. Microarray expression profiles for several genes were verified via northern analysis or quantitative reverse transcription-PCR. We characterized T-DNA insertion mutants for nine chito-oligomer responsive genes. Three of the mutants were more susceptible to the fungal pathogen, powdery mildew, than wild type as measured by conidiophore production. These three mutants included mutants of genes for two disease resistance-like proteins and a putative E3 ligase. The isolation of loss-of-function mutants with enhanced disease susceptibility provides direct evidence that the chito-octamer is an important oligosaccharide elicitor of plant defenses. Also, this study demonstrates the value of microarray data for identifying new components of uncharacterized signaling pathways.

Plants in the environment are constantly under siege by a multitude of disease-causing organisms including bacteria, fungi, viruses, and nematodes. Plants may resist pathogen attack using both preformed defenses (e.g. antimicrobial compounds) and inducible defense responses (for review, see Hammond-Kosack and Jones, 2000; Heath, 2000). Inducible defenses can be activated upon recognition of general elicitors such as bacterial flagellin (Gomez-Gomez and Boller, 2002), the polypeptide systemin (Ryan and Pearce, 1998), and multiple host or pathogen cell wall fragments released during pathogen attack (Nürnberger et al., 2004). Recently, oligosaccharide elicitors such as chito-oligomers and oligagalacturonic acids have received renewed attention as important signals in plant defense responses. The activation of defense genes by these elicitors is thought to be receptor-mediated though little is known about the initial perception and consequent signaling pathways involved in plant cells.

Chito-oligosaccharides can be generated from the cell walls of pathogenic fungi by the action of endochitinases and were shown to elicit strong defense responses in many plant species (Stacey and Shibuya, 1997; Shibuya and Minami, 2001). In a previous study, we showed that transcript levels for 71 expressed sequence tags, representing 61 genes, were altered more than 3-fold in chito-oligomer treated Arabidopsis (Arabidopsis thaliana) seedlings, demonstrating the usefulness of Arabidopsis as a model for studying chitin signaling in plants (Ramonell et al., 2002). Further experimentation by Zhang et al. (2002) showed that this response was not mediated by the well-characterized salicylic acid, jasmonic acid, or ethylene-responsive plant defense pathways. More recently, Wan et al. (2004) demonstrated that the cellular response of Arabidopsis to chitin octamer elicitation involved activation of a mitogen-activated protein kinase cascade. These data indicated that chitin was inducing defense responses through an uncharacterized signaling pathway.

In previous experiments, we used a complex mixture of chito-oligomers derived from hydrolyzed crab-shell chitin (CSC) to induce defense responses in Arabidopsis (Ramonell et al., 2002). However, purified...
chitin fragments (degree of polymerization [dp] = 7–8) induce the synthesis of phytoalexins in suspension cultured rice (*Oryza sativa*) cells at nanomolar concentrations. Yamada et al. (1993) and Zhang et al. (2002) showed that the purified chitin octamer was the most effective of the chito-oligomer lengths tested in inducing the response of several genes identified as responsive to CSC (Ramonell et al., 2002). In this work, we compared the gene expression profiles of seedlings treated with purified chito-octamer (dp = 8) and a CSC mixture using full-genome ATH1 Affymetrix GeneChips. T-DNA insertional mutations in selected chito-oligomer responsive genes were evaluated to determine whether they contribute to disease resistance to the powdery mildew pathogen, *Erysiphe cichoracearum*.

**RESULTS**

**Chitin Is Present at the Tips of Growing Hyphae and in Conidia of E. cichoracearum during Infection of Arabidopsis**

Chitin is one of the major cell wall components in many fungi. Fungal cell walls change during growth and development and the deposition and subsequent removal of chitin is particularly important during septation (Smits et al., 2001; Adams, 2004). However, the distribution of chitin in *E. cichoracearum* structures formed during infections of Arabidopsis was unknown. Cell wall components and chitin were localized in growing *E. cichoracearum* using labeled wheat germ agglutinin (WGA). WGA contains a group of closely related isolecitins that can bind oligosaccharides containing terminal GlcNAc or chitobiose and membrane glycoproteins (Peters and Latka, 1986). WGA has been used in several studies on chitin distribution in fungal walls (Galun et al., 1976; Tronchin et al., 1981). Laser scanning confocal microscopy visualized WGA-488 Alexa conjugates at the growing tips of appressoria and hyphae (Fig. 1, A and B). WGA staining was also observed in the cell walls of conidia developing on conidiophores (Fig. 1C). As the conidia matured and detached from the conidiophore, chitin was localized to both ends of conidia (Fig. 1D). When conidia germinated, the region where the appressorial germ tube developed accumulated chitin. Furthermore, although mature conidia have chitin at their ends (Fig. 1D), germinated conidia (Fig. 1A) lack detectable chitin in this position, suggesting temporal and spatial regulation of chitin localization. Transmission electron microscopy confirmed that WGA-gold colloidal conjugates were present at the growing tip of appressorium (Fig. 1E) and also accumulated in the cell wall of the fungal haustorium, a feeding structure that forms in epidermal cells (Fig. 1F). Fungal structures directly in contact with the plant and likely to be exposed to plant chitinases, such as appressoria and haustoria, show relatively high content of chitin. Therefore, it is likely that chito-oligomers will be generated during the course of powdery mildew infections.

**Figure 1.** Chitin localization in the powdery mildew, *E. cichoracearum*. Confocal images are presented in A to D and transmission electron micrographs in E and F. In A to D, samples were stained with PI (red channel) to highlight fungal structures, although plant structures can be stained with this nonspecific stain, and chitin was localized with the lectin, WGA-Alexa Fluor 488 (green channel). In E and F, WGA-colloidial gold conjugates were used to localize chitin. A, A merged confocal micrograph showing chitin localized to the tip of the appressorium (arrow; 1 dpi). Plant guard cells are also partially stained with PI in this image. Bar = 12 μm. C, Conidium; Ap, appressorium. B, Chitin localization at the growing tip of hypha (arrow) but not on the elongated hyphal region (3 dpi). Hyp, Hyphae. Bar = 11 μm. C, Chitin labeling occurs in the developing conidia still attached to conidiophores (arrows; 7 dpi). Bar = 31 μm. D, A mature conidium (arrow) detaching from a conidiophore shows strong chitin localization at the both ends. Bar = 16 μm. E, Chitin occurs in the fungal appressorial cell wall but not on the plant cell wall. CW, Plant outer epidermal cell wall; FCW, fungal cell wall. Bar = 2.15 μm. F, Chitin is found in the haustorial cell wall (arrows). EHMAT, Extrahaustorial matrix, H, haustorium; Nc, haustorial nucleus. Bar = 9.21 μm.

**Chito-Oligomers Elicit Distinct Gene Expression Profiles in Arabidopsis**

To better understand the responses of Arabidopsis to chito-oligomers, we compared the gene expression...
of seedlings treated with chito-octamer, CSC, or water as a control using Affymetrix ATH1 full-genome microarrays. Of the approximately 23,000 genes represented on the array, 5,012 genes showed altered expression according to the statistical analysis of microarrays (SAM) program with a false discovery rate of ≤5% (Tusher et al., 2001; Fig. 2A). Cluster analysis of genes responsive to CSC and the chito-octamer suggest that these two treatments elicit very similar responses, especially among the induced genes (Fig. 2A). To narrow the list, genes with changes in expression of 1.5-fold or more relative to the water control were selected and then compared among the two chito-oligomer treatments (Fig. 2B). Full lists of the selected genes organized by treatment and induction or repression are provided in supplemental tables (Supplemental Tables I–VI). The chito-octamer uniquely induced 68 genes but the CSC induced a larger set of genes (104). In both treatments, more genes were significantly repressed than induced. Similar numbers of genes were uniquely repressed by both chito-octamer and CSC treatment (238 and 262 respectively). Thus, the relative proportion of genes uniquely repressed by each treatment was larger than the corresponding number of genes induced (Fig. 2B, 1 and 2). Though the overall profiles appear relatively similar at first glance, there are significant differences in the expression profiles between the two chitin treatments.

Figure 2. A, Hierarchical cluster of ratio values (relative to the water control treatment) of 5,435 chito-oligomer responsive genes as determined by the SAM program. Each gene is represented by a single row and each column represents an individual treatment. Magenta represents up-regulated genes; green, down-regulated genes; and black, genes with no change in expression. B, Venn diagrams showing distribution of the subset of these genes showing a ≥1.5-fold change in expression in at least one of the two chito-oligomer treatments relative to the water control. B1, Distribution of 1,252 genes identified as expressed at ≥1.50-fold higher levels in chito-oligomer treated than control samples. B2, Distribution of 1,123 chito-oligomer responsive genes expressed at ≤0.67 of control samples. 8-mer, Treated with chito-octamer; CSC, treated with CSC; Mock, treated with dH2O. Tables with lists of genes in each grouping are presented as supplemental data (Supplemental Tables I–VI).
This may be due to the effect of different chitin species (e.g. chain lengths) in the CSC or due to different relative concentrations, since accurate concentration measurements for a specific oligomer could only be made for the chito-octamer.

The chito-octamer treatment induced genes for several defense-related proteins including pathogenesis-related protein 5, two WRKY transcription factors, and four disease resistance proteins (three of the Toll Interleukin-1 receptor [TIR] or TIR-nucleotide-binding site [NBS]-Leu-rich repeat [LRR] class, and the other an LRR class protein). The largest class of genes repressed in the chito-octamer treatment were unknown or hypothetical proteins, but several interesting genes were repressed 1.5-fold including genes encoding ETR2, an LRR transmembrane protein kinase, the plant defensin protein PDF 2.5, a jasmonate-inducible protein, and several zinc-finger proteins potentially involved in signaling. Treatment with chito-octamer was shown previously to elicit strong defense responses in numerous plant species (Yamada et al., 1993; Zhang et al., 2002). These data indicate that the chitin octamer is inducing a strong defense response in Arabidopsis that is consistent with data previously observed. It is interesting to note that the chito-octamer induced and repressed several genes involved in plant defense. The plant defensin protein PDF 2.5 was down-regulated, while several disease resistance-like proteins and a WRKY transcription factor were stimulated. It is unclear why defense-related genes were repressed by the chitin octamer. One explanation may be that other signals in addition to the presence of chitin may be necessary to elicit an appropriate defense response to a specific pathogen. Large numbers of genes were either induced or repressed (1,002 and 312, respectively) by both CSC and the chito-octamer. Many of the genes induced by CSC and the chito-octamer were defense related, which is expected in light of data from previous studies (Yamada et al., 1993; Ramonell et al., 2002; Zhang et al., 2002). However, there was no clear trend in the distribution of genes that were repressed by both treatments. Clearly, a combination of chito-oligomer fragments elicits a strong defense response, but a clearer understanding of the corresponding repression of genes will require more study.

In the CSC treatment alone, a large number of genes coding for defense-related proteins were induced 1.5-fold including numerous disease resistance-like proteins, WRKY family transcription factors, and an LRR protein kinase. Several cell wall-related transcripts including a subunit of cellulose synthase and a xyloglucan endotransglycosylase were also induced. Genes for several transport proteins (ATP-binding cassette transporter family, integral membrane protein) and some cell wall extensin-related proteins were repressed 1.5-fold in the CSC treatment, as well as genes encoding two disease-resistance proteins, RAR1 (Required for Mla Resistance 1), an LRR protein kinase, and a geranyl-geranyl phosphate synthase involved in the biosynthesis of defensive terpene compounds. Several classes of signaling molecules, protein kinases, and zinc-finger proteins included both induced and repressed genes in the CSC treatment. In several experiments, CSC was shown to induce the strongest changes in gene expression (Zhang et al., 2002) and this pattern is also repeated in our results. The strength of the response to CSC may be a consequence of interactions among multiple lengths of chito-oligomers causing a stronger response in the plant.

Verification of Microarray Data

To validate the changes in gene expression revealed by the microarray experiments, the following categories of genes were selected based on the microarray data for verification tests in northern-blotting experiments (Fig. 3A): (1) four genes up-regulated by CSC and chito-octamer: AtMPK3 (MITOGEN-ACTIVATED PROTEIN KINASE 3 gene, At3g45640), a lectin-like protein gene (At3g16530), ZAT12 (zinc finger protein 12 gene, At5g59820), and a salt tolerance zinc finger protein gene (At1g27730); (2) a gene down-regulated by CSC and the chitin octamer: a putative auxin-regulated protein gene (At2g21200); and (3) a gene not regulated by these treatments: AtERF3 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3 gene, At1g50640). The same RNA samples used in the microarray experiments were used in the northern-blotting analyses, and a gene-specific probe was used for each hybridization. To make a direct comparison between the microarray data and the corresponding northern results, the image was quantified and the fold-change relative to a water control for each gene was calculated with the ACTIN2 gene serving as a loading control. The fold-change values determined from the microarray data (Fig. 3A, white bars) and the northern-blot experiments (Fig. 3A, black bars) correlated well, suggesting that our current microarray data is of good quality.

In addition, the transcript levels of two genes, At2g35000, encoding an E3 ligase-like protein, and At5g25910, encoding a putative disease resistance protein, were determined in independent RNA samples by reverse transcription (RT)-PCR methods. The induction of both genes after treatment with CSC for 30 min was confirmed by RT-PCR (Fig. 3B). Little or no induction of either gene was observed after 15 min of treatment. Quantitative RT-PCR was used to confirm that these two genes were induced by 30 min of treatment with CSC as was observed in the microarray experiments, although the fold-induction values observed in the two experiments differed somewhat (Fig. 3C). The mock value observed for At5g25910 was unusually variable, likely due to the very low expression levels of this gene in mock treated seedlings and the difficulty of reliably measuring these low expression levels.

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Figure 3. Verification of microarrays results for selected genes. A, The top sections present images of the northern blot for each gene. The bottom sections are bar graphs giving the fold-change values for each gene relative to a water control (y axis) for each chito-oligomer treatment (x axis) determined in microarray (white bars) and in northern-blot (black bar) experiments. The ACTIN-2 mRNA (At3g18780) was used as a control in the northern-blot experiments. Genes chosen for northern analysis as follows: AtMPK3, lectin-like protein, ZAT12 and salt-tolerance zinc-finger protein were up-regulated by both the CSC and chito-octamer treatments; the putative auxin-regulated gene was down-regulated by the CSC and chito-octamer treatments and the gene AtERF3 is a gene not regulated by chito-oligomer treatment. B, Transcript levels for At2g35000, encoding an E3 ligase-like gene, and At5g25910, encoding a putative disease resistance protein, at 15 and 30 min after treatment with CSC as determined by RT-PCR. One of three replicates is presented. C, Using the 30-min samples from B, quantitative RT-PCR was used to determine the fold-change in CSC-treated samples relative to water controls. The average and so for three biological replicates is presented. For comparison, averaged data from the three replicates of the microarray experiment for these genes and the so are also shown.
Insertional Mutants in Chito-Oligomer Responsive Genes

From the microarray data, nine genes (At2g28200, At2g02950, At2g34930, At2g35000, At5g25910, At4g11830, At2g41940, At4g16820, and At3g57640) were selected on the basis of their expression profiles for further analysis. A search of the insertion sequence database of the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu) was performed to identify T-DNA insertional mutants with insertions in the nine genes of interest. All lines were screened to identify homozygous mutants. One backcross was performed on all T-DNA insertional mutants with wild-type Columbia (Col-0).

If we assume that chito-oligomer fragments are one of the nonspecific signals or pathogen-associated molecular patterns, exchanged between host and pathogen during fungal and insect attack (Nürnberger et al., 2004) and that plants have evolved a mechanism to detect chito-oligomers and activate defense responses, then one would predict that mutations in chito-oligomer responsive genes would show enhanced susceptibility to pathogens. This prediction also rests on the assumption that a virulent pathogen is not able to significantly suppress or evade the chito-oligomer elicited defenses. Since the chito-octamer and CSC elicited a number of defense-related genes, one would predict that chito-octamer and CSC responsive genes are more likely to play a role in defense. Nine T-DNA insertion lines, one for each selected gene, were inoculated with the fungal pathogen, powdery mildew, and evaluated for disease resistance. To evaluate the levels of susceptibility to powdery mildew, the inoculated T-DNA lines were compared with powdery mildew inoculated Col-0, a susceptible ecotype, Kashmir-1 (Kas-1), a resistant Arabidopsis ecotype, Landsberg erecta (Ler), an ecotype with intermediate resistance to powdery mildew, and two mutant lines impaired in salicylic acid-mediated plant defense that were hyper-susceptible to powdery mildew, the transgenic plant NahG that contains the bacterial gene salicylate hydrolase and the mutant sid2-1 that lacks a gene involved in salicylic acid biosynthesis (Reuber et al., 1998).

At low-density inoculations, two mutants in genes At2g35000 and At5g25910 had more severe macroscopic symptoms compared with Col-0 wild-type plants (Fig. 4A). In contrast, the other seven insertion mutants exhibited a wild-type (Col-0) disease phenotype when challenged by low-density inoculum (data not shown). In addition, there was a clear increase in the growth of fungal hyphae on both mutants (Fig. 4B) compared to growth on Col-0 wild-type plants, though the fungal growth was not as advanced as that on transgenic NahG plants (Fig. 4B). Independent SALK insertion mutants for each of At2g35000 (SALK_066755, SALK_036065, SALK_036066) and At5g25910 (SALK_125444, SALK_107922) showed enhanced disease susceptibility, confirming that the altered disease phenotype can be attributed to the loss of function of At2g35000 and At5g25910 and not to a secondary mutation in the SALK lines (Supplemental Fig. 1). To measure disease development more precisely, conidiophores per colony (c/c) were counted 4 d postinoculation (dpi) and conidiophores per mm$^2$ (c/mm$^2$) leaf surface were counted at 5 dpi for each of the mutants (Table I). Three of the T-DNA insertion lines, At2g34930, At2g35000, and At5g25910, were significantly more susceptible to powdery mildew than Col-0, Ler, and Kas-1 accessions. However, these three mutants were not as susceptible as transgenic NahG plants, which are impaired in the salicylic acid defense signaling and are known to be hypersusceptible to powdery mildew (Reuber et al., 1998; Table I). The other six T-DNA insertional mutants did not show significant changes in disease symptoms compared with Col-0. A pBLAST search on these genes (data not shown) revealed significant numbers of functionally redundant genes present in the Arabidopsis genome that could account for the lack of change in
Table 1. Quantification of E. cichoracearum growth on T-DNA insertion mutants

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<th>Description</th>
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their defense phenotype. Thus, three of the six selected CSC and/or chito-octamer responsive genes showed enhanced disease susceptibility when mutated. The putative gene functions of each of these three genes, two disease resistance proteins, and an E3 ligase are consistent with the hypothesis that these genes may play a role in a chito-oligomer signal transduction pathway leading to the induction of a suite of defense response genes.

DISCUSSION

Chitin fragments may be generated by the action of plant chitinases on fungal or insect cell walls (Boller, 1995) and are known to elicit responses associated with plant defense (Stacey and Shibuya, 1997; Shibuya and Minami, 2001). In a previous study, we demonstrated that Arabidopsis responded to treatment with chito-oligomers by initiating a defense response similar to that of other plant species (Ramonell et al., 2002) and that the chitin response pathway did not appear to be operating through any of the well-characterized defense pathways (i.e. salicylic acid, jasmonate, or ethylene; Zhang et al., 2002). To better understand the chitin response, we treated Arabidopsis seedlings with chito-octamer or CSC and examined expression profiles using full-genome microarrays. The pattern of gene expression elicited by the chito-octamer and the CSC mixture was very similar and included a number of up-regulated defense associated genes. In rice and soybean, longer chitin oligomers, such as the chito-octamer, elicit a variety of defense-related cellular responses (Stacey and Shibuya, 1997; Shibuya and Minami, 2001). Both the chito-octamer and CSC repressed the expression of genes for several defense-related proteins (e.g. RAR1, LRR kinases, PDF 2.5, jasmonate-inducible protein). It is unclear why some defense-associated genes are induced and other genes are repressed by CSC or chito-octamer treatment. CSC consists of an undefined mixture of chito-oligomers of varying lengths up to about chito-octamer (oligosmers with a higher dp are thought to be insoluble in water) and chito-oligomers of different sizes may induce or repress distinct sets of genes. Analogous to the negative cross-talk between the salicylic acid and jasmonate pathways (Dong, 2004), the purified chito-octamer may induce a specific set of defense-related genes and repress other defense-related genes under the regulation of a competing pathway.

A subset of the genes responding to the two chito-oligomer treatments were identified, T-DNA insertional mutations in nine of these genes were obtained and the response of the mutants to the powdery mildew pathogen E. cichoracearum was examined. Of the nine, three mutants were isolated that were more susceptible to powdery mildew: At2g35000 (a putative RING zinc-finger protein), At2g34930, and At5g25910 (putative disease resistance-like proteins). The enhanced disease susceptibility of the mutants in At2g35000 and At5g25910, but not in the remaining seven genes, was also apparent visually following low-density inoculations with the powdery mildew pathogen, suggesting that either these two genes play a larger role in defense against powdery mildew or they more directly influence chitin-activated defense signaling.

The At2g35000 gene product corresponds to a putative RING zinc-finger protein belonging to the Arabidopsis Touxicos en Lavadura (ATL) gene family. Several important Arabidopsis genes with RING domains have been identified including COP1, a photomorphogenesis regulator (Torii et al., 1999), A-RZF, a gene involved in seed development (Zou and Taylor, 1997), and ATL2, which is known to be strongly induced by exposure to chitin and inactivated cellulase preparations and has been characterized as an early-response gene (Salinas-Mondragón et al., 1999). Members of the ATL gene family are postulated to be involved in the early stages of the defense response (Martinez-Garcia et al., 1996), suggesting that At2g35000 may be involved in the initial signaling responses to chitin leading to plant defense.

Both At2g34930 and At5g25910 correspond to two uncharacterized disease resistance-like proteins con-
taining LRR regions. The most widely studied LRR-containing genes involved in plant defense are the plant disease resistance (R) proteins (Martin et al., 2003; Nimchuk et al., 2003). R-genes confer resistance to specific races of pathogens that express the corresponding avirulence (Avr) gene (Dixon et al., 1998). More recently, some LRR-receptor-like kinase proteins have been shown to interact with smaller molecules (e.g. BRI1/brassinosteroid [Kinoshita et al., 2005]; FLS2/Ilg22 peptide [Gomez-Gomez and Boller, 2000]). It is intriguing to speculate that At5g25910 may be acting similarly in chito-oligomer perception when plants are challenged with fungal pathogens like powdery mildew.

**CONCLUSION**

Using genomics techniques, we identified several genes that may play a role in the chitin-mediated defense pathway in Arabidopsis. Our results showed that T-DNA insertions in three genes, At2g35000 (a putative RING zinc-finger protein), At2g34930, and At5g25910 (putative disease resistance-like proteins) resulted in plants that were more susceptible to powdery mildew than wild type, indicating that these genes may play a role in the defense response of Arabidopsis to powdery mildew. Thus, the general elicitor, chitin, has been linked to a defense response in plants indicating that it plays a role in plant responses to fungal pathogens. In addition, this study demonstrates the power of microarray data for identifying potential targets for mutation in uncharacterized signaling pathways. Additional experiments will now be necessary to elucidate the precise functions of these genes in defense responses and chito-oligomer recognition in Arabidopsis.

**MATERIALS AND METHODS**

**Plant Growth Conditions and Chitin Treatments**

For microarray analysis and northern hybridization, Arabidopsis (Arabidopsis thaliana) L. Col-0 seeds were sterilized and grown hydroponically according to Zhang et al. (2002). Briefly, Arabidopsis seeds (approximately 30 seeds/10 mL culture) were surface sterilized and allocated in 50-mL Falcon tubes containing 10 mL of liquid medium (1% Murashige and Skoog basal salt mixture + 2% dextrose, pH 5.8). The tubes were incubated with gentle shaking in a growth chamber with the following settings: constant light at 125 mol m⁻² s⁻¹ at a temperature of 23°C. Gentle shaking was employed to minimize the possible induction of genes by touch or physical injury. After 14 d, chitin was added to the medium containing the seedlings at the following concentrations: hydrolyzed CSC (Sigma-Aldrich, St. Louis) at a final concentration of 100 μmol mL⁻¹ or the purified chito-octamer (dp = 8) at a final concentration of 1 μM. The octamer chitin oligomer was a generous gift from Dr. Naoto Shibuya (National Institute of Agrobiological Resources, Tsukuba, Japan). The control seedlings were similarly treated with an equivalent salt mixture. The control seedlings were similarly treated with an equivalent salt mixture.

**Pathogen Infections**

Three-week-old Arabidopsis plants were inoculated with the powdery mildew pathogen, Erysiphe cichoracearum UCSC1 (Adam and Somerville, 1996). Fungal inoculum was prepared and inoculations performed as described previously (Wilson et al., 2001). After all inoculations, plants were placed in a dew chamber in the dark at 100% relative humidity for 1 h and then were placed into a growth chamber under conditions described previously.

**Light Microscopy and Photography Techniques**

To visualize fungal hyphae, infected leaves were cleared in ethanol for 2 h and then stained with 250 μg/mL trypan blue in a solution of lactic acid, glycerol, and water (1:1:1) for 15 min and mounted with 60% glycerol solution (v/v) on microscope slides. Individual colonies were photographed using a Nikon Eclipse E600 camera and images were analyzed with the Spot Advance 32 program attached to a Leica microscope. The size of the microscopic image was calculated using a microscopic ruler in order to quantify the numbers of conidiophores per millimeter squared. A Student’s t test was performed using Excel (Microsoft, Seattle) to identify ratios that were significantly different from those in wild-type plants. Both conidiophores/colony and conidiophores per millimeter squared were measured. Photographs of whole plants and infected leaves were taken using a Nikon Coolpix E995 digital camera.

**Electron Microscopy**

For electron micrographs, Arabidopsis leaves infected for 24 h with E. cichoracearum UCSC1 were cut into small pieces and fixed in 3% glutaraldehyde (0.05 M phosphate buffer, pH 6.8) for 1.5 h. Tissue was rinsed in buffer and post fixed in 1% OsO₄ in buffer for 2 h, rinsed in buffer, dehydrated in an ethanol series, and embedded in Spurr’s medium. Thin sections were cut on a Reichert ultramicrotome, picked up on gold-gilded grids and floated on 0.1 M sources. T-DNA insertion lines were sequenced and mapped at the SALK Institute (Alonzo et al., 2003; Yamada et al., 2003). For infection studies, seeds of Col-0, Kas-1, Ler, transgenic NahG g transgenic plants, and T-DNA insertion mutants were placed on soil, cold treated for 6 d, and placed into a growth chamber. Plants were grown on soil for 3 weeks in a growth chamber at 60% (v/v) humidity at temperatures of 24°C (day) and 22°C (night), with a photoperiod of 14 h light/10 h dark, and a light intensity of 150 μmol m⁻² s⁻¹ for all experiments on soil. After 3 weeks, plants were inoculated with the powdery mildew fungus (see pathogen infections) and placed in a chamber at the same light and temperature conditions except with 80% humidity. Chitin treatments of seedlings for the RT-PCR analysis were as follows: seedlings were surface sterilized using 75% ethanol followed by 50% bleach and a water rinse. Approximately 500 seeds (10 mg) were grown per Erlenmeyer flask in 125 mL of Murashige Skoog medium (Sigma). At 2 g/L, pH 5.7 supplemented with Gamborg vitamins (Sigma). Erlenmeyer flasks with the seeds were incubated in the cold for 6 d and then were placed in a shaking incubator at 150 rpm for 3 weeks under constant illumination (125 μmol m⁻² s⁻¹) at 23°C. After 14 d, hydrolyzed CSC (Sigma) was added to the medium containing the seedlings at a final concentration of 100 μg/mL. Whole seedlings were collected 30 min after treatment, immediately frozen in liquid N₂, and stored at −80°C for later use.

**Chitin Localization Using Confocal and Transmission Electron Microscopy**

For experiments performed using confocal microscopy, 3-week-old Arabidopsis (Col-0) was infected with E. cichoracearum UCSC1 and cut rosette leaves were stained with WGA-AlexaFluor488 conjugate (Molecular Probes, Eugene, OR) at a concentration of 10 μg/mL (w/v) in 1× phosphate-buffered saline (PBS), pH 7.4, and 0.1% silwet for an hour (up to 24 h) at room temperature. Leaves were then washed twice in a solution of 1× PBS, pH 7.4, and 0.01% silwet. The WGA stained and washed leaves were counterstained with propidium iodide (PI, 10 μg/mL) in the same buffer used for both WGA stain and wash. PI stained fungal structures and WGA-AlexaFluor488 stained chitin were visualized simultaneously by Bio-Rad MRC 1024 laser scanning confocal microscope at 568 nm and 488 nm (excitation by Ar/Kr laser) and 598 nm and 522 nm (emission), respectively (Bio-Rad, Hercules, CA). Z-series images were acquired by Lasersharp (Bio-Rad) and merged in Conocal Assistant (version 4.02, Bio-Rad).

For electron micrographs, Arabidopsis leaves infected for 24 h with E. cichoracearum UCSC1 were cut into small pieces and fixed in 3% glutaraldehyde (0.05 M phosphate buffer, pH 6.8) for 1.5 h. Tissue was rinsed in buffer and post fixed in 1% OsO₄ in buffer for 2 h, rinsed in buffer, dehydrated in an ethanol series, and embedded in Spurr’s medium. Thin sections were cut on a Reichert ultramicrotome, picked up on gold-gilded grids and floated on 0.1 M sold.
Northern Hybridizations

Total RNA was isolated from each sample using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction and was purified further using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA).

For northern hybridizations, 10 µg of total RNA was separated on a denaturing, formaldehyde, 1% agarose gel and vacuum blotted onto Zeta-Probe GT blotting membrane (Bio-Rad Laboratories, Hercules, CA). Gene-specific probes were made with a Prime-a-Gene Labelling System (Promega, Madison, WI) from the genes listed below. Mitogen-activated protein kinase 3 (AtMPK3) gene (At3g54640), a lectin-like protein gene (At3g16530), and ACTIN-2 (At3g18780) were from Zhang et al. (2002). The following genes were amplified from the genomic DNA by their forward and reverse primers: zinc finger protein (Zat12) gene (At3g59820), LIPOLYTIC TRANSFAESE (At1g06460), ethylene responsive element binding factor 3 (ERF3) gene (At1g50640), salt tolerance zinc finger protein gene (At1g27730), and a putative auxin-regulated protein gene (At2g21210). Primer sequences and probe lengths are given in Supplemental Table VII. The amplified sequences were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and confirmed by sequencing. Prehybridization, hybridization, washing, and stripping were conducted according to the manufacturer’s instructions (Bio-Rad). Images were captured using FujiFilm FLA-5000 imaging system (Fujifilm Medical Systems USA, Stamford, CT) and quantified using the software FUJIFILM Science Lab 99 Image Gauge (Ver. 3.4; FujiFilm Medical Systems USA). ACTIN-2 was used as a loading control. The relative fold change for each gene was determined using the calculation: (signal intensity of the gene in the treated sample/signal intensity of ACTIN-2 in the treated sample)/ (signal intensity of the gene in the control sample/signal intensity of ACTIN-2 in the control sample).

PCR and RT-PCR

Genomic DNA for PCR reactions was extracted using the method described by Dellaporta et al. (1983). For RT-PCR, total RNA was isolated from frozen tissue using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Trace amounts of genomic DNA were removed by digestion with DNase (DNA-free, Ambion, Austin, TX, or RQI DNase, Promega), first-strand cDNA synthesis was primed with oligo(dT)12-18 anchor primer; with the First-Strand Synthesis kit (Amersham-Pharmacia, Rainham, UK) according to the manufacturer’s protocol. An aliquot of 1.5 µL was used as template for PCR. For nested PCR of T-DNA insertion mutants, 1 µL of 1/50 dilution from the first PCR was used as template. The RT-PCR, PCR, and nested PCR program consisted of 3 min at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C. The final extension step consisted of 7 min at 72°C. PCR fragments amplified were visualized on 1.5% agarose gels.

Quantitative RT-PCR

Quantitative RT-PCR experiments were done using the SYBR Green qPCR kit (Finnzymes, Espoo, Finland) at a final volume of 20 µL with the cycle protocol recommended by the manufacturer. Samples were run using a DNA Engine Option 2 system with a PTC-200 DNA Engine Cycler and a CFD-3220 Option 2 detector (MJ Research, Waltham, MA). Reactions were carried out using 1X SYBR Green concentration and 1X SYBR Green master mix, with a sample as a template. PCR cycling conditions were composed of an initial step at 95°C for 3 min followed by 94°C for 10 s and 62°C for 20 s, 72°C for 24 s before the first plate reading, and a step of 82°C for 1 s, before the second reading, during 40 cycles. All cDNA samples were assayed in triplicate. Data from the quantitative RT-PCR was analyzed using Option Monitor Analysis software (version 2.01, MJ Research), and Microsoft Excel (Microsoft). Statistical analysis of the data was done as described by Livak and Schmittgen (2001) using the 2-ΔΔCT method. For the analysis of At2g35000 and At5g25910 genes, a Student’s t test was performed to identify induction ratios (CSC treated/ water treated) that were significantly different from a value of 1. Gene-specific primers were obtained using the Oligo 4.0 program (Scientific & Educational Software, Cary, NC) and used for RT-PCR and quantitative RT-PCR. PCR primers were designed for optimal amplification between 62°C to 65°C and to generate an amplicon of about 300 bp in length. Specific primer sequences were: ACTIN-2 (At3g18780), forward primer 5’-GTGTGTTAGAAGGCA- CAATCCAG-3’ and reverse primer 5’-CTGGAACAGACTTGGCTT- CATC-3’; At2g35000, forward primer 5’-GTCCAGAGATTCTTCGCGG- CATCC-3’ and reverse primer 5’-CAGCCGACATCTGTAATACAC-3’; and At5g25910, forward primer 5’-CCCGCCTTCGAGTACAACG-3’ and reverse primer 5’-GGAATGCGCTGGTTGTAATAGG-3’.

Characterization of the T-DNA Insertion Lines

The location of the T-DNA insertions within each line was originally identified at the Salk Institute Genomic Analysis (La Jolla, CA) and confirmed using the protocols described by Siebert et al. (1995). The SALK insertion lines used in this study were: SALK_048250, SALK_005340, SALK_094846, SALK_066755, SALK_045674, SALK_017220, SALK_089965, SALK_013856, SALK_054976, SALK_050666, SALK_125444, SALK_107922, and SALK_036065. Twelve seeds of each T-DNA insertion line were grown as described previously; samples from each plant were taken and genomic DNA extraction was performed as described by Dellaporta et al. (1983). PCR analysis was performed according to the protocols recommended by SALK Institute (http://signal.salk.edu/tdna_protocols.html). For the first round of PCR to confirm the T-DNA insertions, primers were designed to verify the T-DNA insertion using the SIGMAX Setool Toolbox program (http://signal.salk.edu/ dnaprimers.html). After the primary PCR reaction, 1 µL of 1:50 dilution from the first PCR was used for a second round of nested PCR. Homozygous individuals identified from the nested PCR were allowed to self-fertilize and their progeny subjected to an additional round of nested PCR. Individuals, that passed the second screen, were allowed to self and their progeny were collected and used for the experiments described in this paper. One backcross was performed on all T-DNA insertion lines tested. All appropriate seed stocks will be deposited at the ABRC at Ohio State University. Lists of primers used in this study are provided in Supplemental Tables VIII, IX, and X.

Microarray Preparation, Hybridization, and Data Extraction

Total RNA samples were processed according to manufacturer’s protocols with the following modifications (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix, Santa Clara, CA). Single-stranded, double-stranded cDNA was synthesized from the poly(A) + mRNA present in the isolated total RNA (20 µg of total RNA starting material for each sample read) using the SuperScript III Double-Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA) and custom poly (T) (nucleotide primers that contained a sequence recognized by T7 RNA polymerase). All of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction, using the Bio-Array High-Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY). Twelve micrograms of the resulting biotin-tagged cRNA was fragmented to strands of less than 100 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). The fragmented target cRNA (20 µg) was hybridized at 45°C with rotation at 60 rpm for 16 h (Affymetrix GeneChip Hybridization Oven 640) to probe sets present on an Affymetrix ATH1 GeneChip array. The GeneChip arrays were washed and then stained (streptavidin-phycocerythrin) on an Affymetrix Fluidics Station 400, followed by scanning on a Hewlett-Packard GeneArray scanner (Hewlett-Packard, Palo Alto, CA). Image analysis and pixel intensity were quantified using MicroArray Suite 5.0 software (Affymetrix). Text files were then generated and exported into GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA) for normalization and further analysis. Three biological replicates were performed per treatment.

Microarray Data Analysis

Text files containing raw data were imported into GeneSpring 6.0 (Silicon Genetics, Redwood City, CA) and were normalized as follows. First, values below 0.01 were set to 0.01. Each measurement was then divided by the 50th percentile of all measurements in that sample. Specific samples were then normalized to one another: samples 1 to 9 (CSC, chito-octamer, and water treatment, three replicates of each treatment) were normalized against the
median of the control samples (water treatment). Each measurement for each gene in those specific samples was divided by the median of that gene’s measurements in the corresponding control samples.

These data on all genes were then extracted and analyzed for significance using the SAM software (Tusher et al., 2001). Genes determined to be statistically significant were listed and the resulting information was imported into GeneSpring 6.0 for further analysis. Genes were grouped by their biological function via their gene ontology annotation using the gene ontology annotation search, functional characterization download function on the Arabidopsis Information Resource Web site (TAIR, www.arabidopsis.org). Expression data sets have been deposited in the Gene Expression Omnibus (GSE25338) at the National Center for Biotechnology Information (Edgar et al., 2002; www.ncbi.nlm.nih.gov/geo/).

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LITERATURE CITED


