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Pathogen-Induced Systemic Activation of a Plant Defensin Gene in Arabidopsis Follows a Salicylic Acid-Independent Pathway

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A 5-kD plant defensin was purified from Arabidopsis leaves challenged with the fungus *Alternaria brassicicola* and shown to possess antifungal properties in vitro. The corresponding plant defensin gene was induced after treatment of leaves with methyl jasmonate or ethylene but not with salicylic acid or 2,6-dichloroisonicotinic acid. When challenged with *A. brassicicola*, the levels of the plant defensin protein and mRNA rose both in inoculated leaves and in nontreated leaves of inoculated plants (systemic leaves). These events coincided with an increase in the endogenous jasmonic acid content of both types of leaves. Systemic pathogen-induced expression of the plant defensin gene was unaffected in Arabidopsis transformants (*nahG*) or mutants (*npr1* and *cpr1*) affected in the salicylic acid response but was strongly reduced in the Arabidopsis mutants *ein2* and *coi1* that are blocked in their response to ethylene and methyl jasmonate, respectively. Our results indicate that systemic pathogen-induced expression of the plant defensin gene in Arabidopsis is independent of salicylic acid but requires components of the ethylene and jasmonic acid response.

INTRODUCTION

It is well documented that plants challenged by microbial pathogens are able to induce the expression of a set of defense-related genes, such as the genes encoding pathogenesis-related (PR) proteins. These genes are expressed locally as well as in distant, noninfected leaves (Linthorst, 1991; Ward et al., 1991; Uknes et al., 1992). Accumulation of PR proteins coincides with a plant's enhanced resistance to subsequent challenge by microbial pathogens. This phenomenon is known as systemic acquired resistance (SAR; Ryals et al., 1994).

Salicylic acid (SA) has been shown to play an important role in the signal transduction pathway leading to SAR. Infection of leaves by microbial pathogens causes an increase in the endogenous levels of SA followed by the induction of PR proteins both locally and systemically and by the onset of SAR (Malamy et al., 1990; Métraux et al., 1990). Exogenous application of SA also results in the accumulation of PR proteins and in the establishment of SAR (Métraux et al., 1990; Ward et al., 1991; Uknes et al., 1992). Compelling evidence linking SA to SAR came from experiments with transgenic tobacco and Arabidopsis plants expressing the *nahG* gene of *Pseudomonas putida*. *nahG* encodes salicylate hydroxylase, which converts SA to catechol. These plants are unable to accumulate significant levels of SA and do not develop SAR when infected with a pathogen or treated with SA (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995).

Although SA is clearly implicated in SAR, it remains to be determined whether SA itself is the long-distance signal that originates at the infection site and moves throughout the plant. Following pathogen attack, cucumber plants are able to trigger SAR even if the inoculated leaves were removed before any significant accumulation of SA could be detected, thus indicating that a signal other than SA may also be required for the onset of SAR (Rasmussen et al., 1991). In addition, chimeric tobacco plants consisting of transgenic *nahG*-expressing rootstocks and untransformed scions display SAR in the upper wild-type leaves after pathogen infection of transgenic lower leaves expressing *nahG* (Vernooij et al., 1994). On the other hand, Shulaev et al. (1995) showed that at least 60% of the SA accumulating in nontreated tobacco leaves from infected plants originates from the lower infected leaf, thus providing evidence in favor of SA being the translocated signal for SAR.

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The gaseous plant hormone ethylene has also been proposed to play a role in defense responses. Pathogen challenge often causes an increase in ethylene production (Ross and Williamson, 1951; Mauch et al., 1984; Boller, 1991), and exogenous application of ethylene induces defense-related enzymes such as glucanases and chitinases (Boller et al., 1983; Mauch and Staehelin, 1989) as well as enzymes involved in phytoalexin synthesis (Ecker and Davis, 1987). However, ethylene is believed to be a symptom rather than a signal of the defense response, because infected plants treated with inhibitors of ethylene biosynthesis are still capable of producing glucanases and chitinases (Boller et al., 1983). In support of this idea, an Arabidopsis mutant insensitive to ethylene was able to mount an SAR response, suggesting that ethylene is not required for the onset of SAR (Lawton et al., 1995). Nevertheless, ethylene could be involved in the SA-dependent defense response by enhancing tissue sensitivity to low levels of SA (Lawton et al., 1994).

Another signal molecule implicated in plant defense responses is jasmonic acid. Exogenous application of this hormone or its analog methyl jasmonate causes induction of different plant defense-related proteins, such as thionins in barley leaves and Arabidopsis seedlings (Andresen et al., 1992; Epple et al., 1995, respectively), proteinase inhibitors in tomato (Farmer and Ryan, 1992; Farmer et al., 1992), and enzymes involved in phytoalexin biosynthesis in cell cultures of different plants (Gundlach et al., 1992; Mueller et al., 1993). In addition, treatment of potato and tomato plants with jasmonic acid results in local and systemic protection against *Phytophthora infestans* (Cohen et al., 1993). Additional support for a role for jasmonic acid in disease resistance is based on the observation that wounding of intact plants or treatment of cell cultures with fungal elicitors enhances the biosynthesis of jasmonic acid (Creelman et al., 1992; Gundlach et al., 1992). Although the role of methyl jasmonate and jasmonic acid in pathogen-induced SAR is currently unclear, it has been hypothesized that these plant metabolites act as secondary messengers in an analogous systemic response induced by wounding (Ryan, 1992). The signal for the wound-induced systemic induction of protease inhibitors is systemin, a mobile peptide released at wound sites, and it is believed that systemin stimulates the release of jasmonic acid in remote tissues (Ryan, 1992).

In this study, we have investigated the induction of a plant defensin gene in Arabidopsis when the plant is attacked by the pathogen *Alternaria brassicicola*. Plant defensins are a family of cysteine-rich basic proteins ~5 kD in size that are structurally related to the antimicrobial insect defensins found in various insect species (Broekaert et al., 1995). A number of plant defensins are potent inhibitors of fungal growth (Terras et al., 1992, 1993, 1995; Moreno et al., 1994; Osborn et al., 1995), suggesting that they may play a role in plant host defense. Indirect evidence for the involvement of plant defensins in host defense has been obtained from the analysis of transgenic tobacco plants that constitutively express a plant defensin from radish seed (Rs-AFP2). The leaves of these transgenic plants display a significantly decreased susceptibility to infection by the fungus *A. longipes* compared with untransformed plants (Terras et al., 1995). In radish, two antifungal plant defensins (Rs-AFP1 and Rs-AFP2) are located predominantly in the outer cell layers of seed organs and are preferentially released during seed germination, thus creating a zone around the germinating seed in which fungal growth is suppressed (Terras et al., 1995). Two additional plant defensins (Rs-AFP3 and Rs-AFP4) are produced in radish leaves when challenged by fungal pathogens. The induced accumulation of plant defensins in radish leaves is not restricted to the infected leaves but also occurs in noninfected leaves (Terras et al., 1995). We now show that an Arabidopsis analog of the radish leaf plant defensin genes is systemically induced when attacked by a pathogen. This induction is not mediated by SA or by signal transduction components of the SA response pathway but rather requires functional components of the ethylene and jasmonic acid response pathways.

RESULTS

Differential Expression of Two Arabidopsis Genes Encoding Homologous Plant Defensins

A search of the GenBank data base for sequences homologous to the nucleotide sequence of the cDNA for Rs-AFP1, which is one of the radish plant defensins (Terras et al., 1995), revealed two Arabidopsis sequences with GenBank accession numbers Z27258 and T04323, respectively (Figures 1A and 1B). These sequences were identified as expressed sequence tags (ESTs) in cDNA libraries prepared from either dry seed mRNA (Z27258) or seedling mRNA (T04323). The genes corresponding to Z27258 and T04323 encode preproteins with a putative signal peptide and a mature plant defensin domain that is 98 and 92% identical to Rs-AFP1 from radish, respectively (Figure 1C). The mature plant defensin domain of the protein encoded by Z27258 has the same N-terminal sequence as an antifungal plant defensin that we had previously isolated from Arabidopsis seed (Terras et al., 1993), except that the amino acid at position 4 of the mature protein domain of the Z27258 encoded protein is predicted to be a phenylalanine, whereas it is a cysteine in the protein purified from seed. Nucleotide sequence analysis of a gene corresponding to Z27258 revealed that the fourth codon of the mature protein domain is TGC (cysteine) and not TTC (phenylalanine) as in Z27258 (J.M. Manners, unpublished results), indicating that the codon in the latter is probably erroneous. The genes corresponding to Z27258 and T04323 are referred to in this article as *PDF1.1* (for plant defensin) and *PDF1.2*, respectively.

The expression pattern of *PDF1.1* and *PDF1.2* was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) with DNase-treated total RNA isolated from different Arabidopsis organs (Figures 2A and 2B). As a control for the RT-PCR

A

PDF1.1

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1       AAACAACTAAGTATAAACAATAGTCATGGCTAAGTCTGCTACCATCGTTACTCTTTTCTTCGCTGCT
-29                                 M  A  K  S  A  T  I  V  T  L  F  F  A  A

68      CTTGTTTTCTTTGCTGCTCTTGAAGCACCGATGGTGGTGGAAGCACAGAAGTTGTGCGAGAGGCCAAGT
-15      L  V  F  F  A  A  L  E  A  P  M  V  V  E  A  Q  K  L  C  E  R  P  S

137     GGGACATGGTCCGGAGTTTGCGGAAACAGTAACGCGTGCAAGAATCAGTGCATTAACCTTGAGAAAGCA
+9       G  T  W  S  G  V  C  G  N  S  N  A  C  K  N  Q  C  I  N  L  E  K  A

206     CGACATGGATCTTGCAACTATGTCTTCCCAGCTCACAAATGTATCTGCTACTTCCCATGTTAATCTACC
+32      R  H  G  S  C  N  Y  V  F  P  A  H  K  C  I  C  Y  F  P  C

275     AAGAGCTCTTAATGCTTAATTATAAAGTGTGTATTTCTATTAAATAAGTATGTGTCACTCTATGAGAGG

344     TCTTATGACATGTACCAGATATGTTATGTTGCTTTGTTTTAATAAGGTAAACTTTTACGG
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B

PDF1.2

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1       ATACATTGAAAACAAAATAGTAATAATCATCATGGCTAAGTTTGCTTCCATCATCACCCTTATCTTC
-29                                 M  A  K  F  A  S  I  I  T  L  I  F

68      GCTGCTCTTGTTCTCTTTGCTGCTTTCGACGCACCGGCAATGGTGGAAGCACAGAAGTTGTGCGAGAAG
-17      A  A  L  V  L  F  A  A  F  D  A  P  A  M  V  E  A  Q  K  L  C  E  K

137     CCAAGTGGGACATGGTCAGGGGTTTGCGGAAACAGTAATGCATGCAAGAATCAGTGCATTAACCTTGAA
+7       P  S  G  T  W  S  G  V  C  G  N  S  N  A  C  K  N  Q  C  I  N  L  E

206     GGAGCCAAACATGGATCATGCAACTATGTCTTCCCAGCACACAAGTGTATCTGTTACGTCCCATGTTAA
+30      G  A  K  H  G  S  C  N  Y  V  F  P  A  H  K  C  I  C  Y  V  P  C

245     ATCTACCACTAATCTTTGGTGCTAAATCGTGTGTATTTTACATAAAAATAAGTCNCTGTCACTCTNTGA

314     GTAACTTTATGACATGCATATTTCTGGTTTTAATTGNTTATTTTNCCGCCGGTTGGT
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C

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PDF1.1    QKLCERPSGTWSGVCGNSNACKNQCINLEKARHGSCNYVFPAHKCICYFPC
PDF1.2    QKLCEKPSGTWSGVCGNSNACKNQCINLEGAKHGSCNYVFPAHKCICYVPC
Rs-AFP1   QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC
Rs-AFP3    KLCERSSGTWSGVCGNNNACKNQCIRLEGAQHGSCNYVFPAHKCICYFPC
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Figure 1. Nucleotide Sequences and Deduced Amino Acid Sequences of Expressed Sequence Tags Z27258 and T04323, Corresponding to *PDF1.1* and *PDF1.2*, Respectively.

(A) Nucleotide sequence and deduced amino acid sequence of Z27258, corresponding to *PDF1.1*. The experimentally determined N-terminal region of PDF1.1, previously called At-AFP1 (Terras et al., 1993), is underlined. The nucleotide at position 123 (T residue in Z27258) has been replaced by a G residue due to additional sequence data.
(B) Nucleotide sequence and deduced amino acid sequence of T04323, corresponding to *PDF1.2*.
(C) Alignment of the amino acid sequences of mature Rs-AFP1 and Rs-AFP3 from radish seed and leaves, respectively (Terras et al., 1995; F.R.G. Terras, unpublished results), with the mature domain of PDF1.1 and the putative mature domain of PDF1.2.
Underlined nucleotide sequences in **(A)** and **(B)** correspond to the positions of primers used for reverse transcriptase–polymerase chain reaction. Stop codons in **(A)** and **(B)** are double underlined.

reactions, a primer pair was designed for amplification of sequences corresponding to the region of the Arabidopsis *ACTIN-1* gene (Figure 2C; Nairn et al., 1988) encompassing a 99-bp intron. In this way, products derived from PCR amplification of genomic DNA could be distinguished by size from true RT-PCR products.

As shown in Figure 2, RNA samples from all analyzed tissues, except dry seed, yielded *ACTIN-1* RT-PCR amplification products of the expected size, whereas genomic DNA yielded a PCR product that was ~100 bp longer. RT-PCR with a primer pair specific for *PDF1.1* resulted in amplification products when RNA isolated from siliques and dry seed were used as templates. The primer pair specific for *PDF1.2* did not yield RT-PCR amplification products in any tissue analyzed from healthy plants. However, amplification products of the expected size were detected by RT-PCR performed with RNA from leaves inoculated with an isolate of *A. brassicicola* that was incompatible with the Columbia (Col-0) ecotype of Arabidopsis and produced limited brown necrotic lesions. The PCR amplification product obtained with genomic DNA was ~100 bp longer than that obtained by RT-PCR with RNA from infected leaves, indicating that the *PDF1.2*-specific primers span an intron in the *PDF1.2* gene. This was not observed with *PDF1.1*-specific primers.

We concluded that Arabidopsis contains two genes that encode highly homologous plant defensins but that have a totally different expression pattern. *PDF1.1* is expressed predominantly in seed and is considered to be the analog of the radish *Rs-AFP1* and *Rs-AFP2* genes, whereas *PDF1.2* is expressed in leaves upon pathogen-induced stress and can be considered the analog of the *Rs-AFP3* and *Rs-AFP4* genes from radish (Terras et al., 1995).

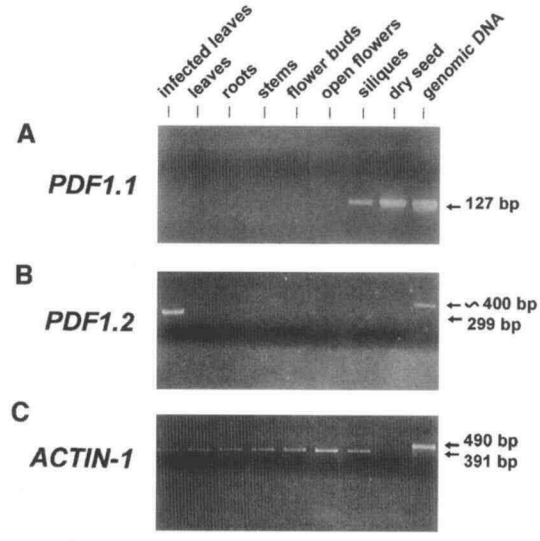

Figure 2. Expression Pattern of *PDF1.1* and *PDF1.2* in Arabidopsis.

(A) RT-PCR analysis using a primer pair specific for *PDF1.1*.
(B) RT-PCR analysis using a primer pair specific for *PDF1.2*.
(C) RT-PCR analysis using a primer pair specific for *ACTIN-1*.
RT-PCR reactions were performed on DNase-free total RNA isolated from different Arabidopsis organs. Roots, stems, flower buds, open flowers, and siliques were collected from 7-week-old flowering plants. Leaves and infected leaves were collected from 4-week-old plants. Infected leaves were inoculated with *A. brassicicola* and collected after 3 days of incubation. Genomic DNA was isolated from leaves of 4-week-old plants and, unlike the RNA samples, was not DNA treated. Numbers at right indicate the lengths of the PCR products.

A Plant Defensin with Antifungal Activity Accumulates in Pathogen-Stressed Arabidopsis Leaves

To investigate whether the pathogen-induced plant defensin in Arabidopsis leaves is biologically active, we attempted to purify the protein by a method previously developed for the purification of Rs-AFP3 and Rs-AFP4 from fungus-infected radish leaves (Terras et al., 1995). In short, the purification method consists of passage of a crude leaf protein extract over an anion exchange column at pH 7.5, passage of the unbound proteins over a cation exchange column at pH 5.5, elution of the bound proteins at high ionic strength, and finally, separation of the eluted proteins over a reversed-phase chromatography column. A comparison of the reversed-phase chromatogram of proteins from leaves of noninoculated Arabidopsis plants with that of proteins from leaves inoculated with *A. brassicicola* led to the identification of a peak present only in the latter (Figure 3A, peak 1). SDS-PAGE analysis showed that the protein in this peak fraction runs as a single 5-kD band comigrating with Rs-AFP1 (Figures 3B and 3C). This protein was equally well recognized as Rs-AFP1 on immunoblots prepared from SDS–polyacrylamide gels and developed with anti–Rs-AFP1 antiserum (Figure 3C). Furthermore, the protein in the peak 1 fraction was demonstrated to inhibit the growth of *A. brassicicola* in vitro (Figure 3D) as well as the growth of *Fusarium culmorum* (results not shown). The growth inhibition was characterized by hyphal hyperbranching and tip swelling, as observed for Rs-AFP1 (Figure 3D) and related plant defensins from other Brassicaceae, including PDF1.1 (previously called At-AFP1) from Arabidopsis seed (Terras et al., 1993).

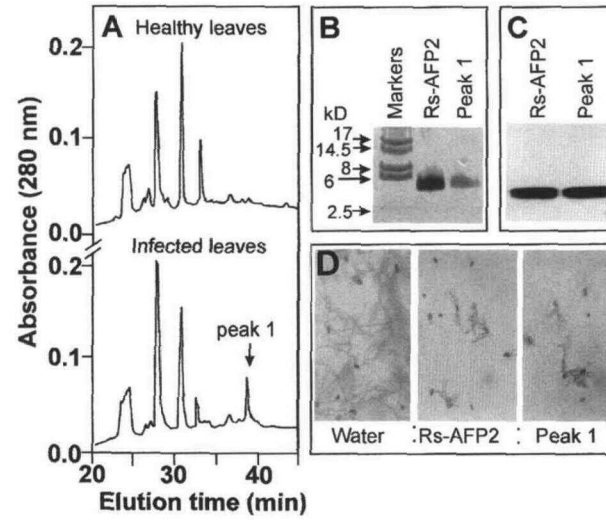

Figure 3. Purification and Characterization of a Plant Defensin from Infected Arabidopsis Leaves.

(A) Separation of the basic protein fraction of healthy Arabidopsis leaves (top) and *A. brassicicola*–infected Arabidopsis leaves (bottom) on a C2/C18 silica reversed-phase chromatography column. The column was eluted with a linear gradient from 0 to 50% (v/v) of acetonitrile in 0.1% (v/v) trifluoroacetic acid.

(B) Electrophoretic analysis of proteins contained in peak 1. Two hundred nanograms of protein contained in peak 1 and 200 ng of purified Rs-AFP2 were electrophoresed on an SDS–polyacrylamide gel (PhastGel High Density) and stained with silver. Sizes of the molecular mass markers are indicated at left in kilodaltons.

(C) Immunoblot of proteins contained in peak 1 after SDS-PAGE, using anti–Rs-AFP1 antibodies. Lanes were loaded with either 200 ng of purified Rs-AFP2 or 200 ng of purified protein contained in peak 1.

(D) In vitro antifungal activity of water (negative control, left), purified Rs-AFP2 at 5 μg/mL (center), or 5 μg/mL purified protein contained in peak 1 (right) against *A. brassicicola*. Micrographs were taken after 24 hr of incubation at 22°C.

Induction of Plant Defensins in Arabidopsis Plants Treated with Chemicals

To date, most of the genes studied that are induced by pathogen stress can be induced at least partially by SA or 2,6-dichloroisonicotinic acid (INA), a synthetic compound mimicking the action of SA (Ward et al., 1991). Whereas the *PR-1* gene was strongly induced by these chemicals, as shown by RNA gel blot analysis, no induction of plant defensin gene expression could be observed upon treatment with SA or INA, whether assessed by RNA gel blot analysis or by ELISA (Figures 4A and 4B). In contrast, application of ethylene or methyl jasmonate significantly increased the level of plant defensin gene expression but did not affect *PR-1* mRNA steady state levels (Figure 4A). No increase in plant defensin gene expression was detected in the appropriate control treatments, that is, incubation in air (control for ethylene) or application of 0.1% (v/v) ethanol (solvent control for methyl jasmonate).

The chemicals paraquat and rose bengal were also tested for their effect on expression of plant defensin genes and *PR-1*. Treatment of plants with paraquat or rose bengal is known to result in the formation of the reactive oxygen species superoxide anion and singlet oxygen, respectively (Bowler et al., 1992; Green and Fluhr, 1995), and hence to cause oxidative stress. Plant defensin protein and mRNA were strongly induced by either paraquat or rose bengal, whereas *PR-1* mRNA was not (Figure 4). Taken together, these data indicate that plant defensin genes and *PR-1* are induced by different groups of chemicals.

Wounding of Arabidopsis leaves did not result in accumulation of detectable levels of either *PR-1* or plant defensin mRNA when assessed 48 hr after treatment (Figure 4). Because a number of wound-induced genes are known to be "switched on" transiently at relatively short time periods after treatment (Warner et al., 1993; Berger et al., 1995), we also verified plant defensin gene expression, both by RNA gel blot analysis and ELISA, at 3, 6, 9, 12, 24, and 48 hr after wounding. However, expression of plant defensin genes could not be detected at any of the analyzed time points, irrespective of whether wounding was applied by making incisions in the leaves with a scalpel or by crushing the leaves with forceps (results not shown).

Simultaneous Systemic Accumulation of Plant Defensins and Jasmonic Acid in Arabidopsis Plants Subjected to Pathogen Stress

We have previously shown that plant defensin genes in radish leaves are systemically induced when locally infected with *A. brassicicola* (Terras et al., 1995). To investigate whether this is also the case for Arabidopsis, we analyzed the induction of plant defensins in Arabidopsis leaves inoculated with *A. brassicicola* and nontreated leaves of inoculated plants (systemic leaves) by using both ELISA (using antigen affinity-purified anti–Rs-AFP1 antiserum) and RNA gel blot analysis (using *PDF1.2* as a probe for defensins). Plant defensins were found to increase from amounts below the detection limit (0.05 μg/mg total soluble protein) at the sampling time just before inoculation to up to 10 and 3 μg/mg total soluble protein in the pathogen-treated leaves and nontreated, systemic leaves at 72 hr after inoculation, respectively (Figure 5A). Steady state levels of plant defensin mRNA rose after inoculation both in pathogen-treated and nontreated, systemic leaves, and the mRNA amounts in both of these leaf samples reached a maximum at 48 hr after inoculation (Figure 5B).

The levels of endogenous jasmonic acid were measured at different time points after inoculation of leaves with *A. brassicicola*, both in inoculated leaves and in noninoculated, systemic leaves (Figure 5C). In inoculated leaves, jasmonic acid levels started to increase 24 hr after inoculation, reaching a maximum after 72 hr, which was >50-fold higher relative to the level in mock-inoculated leaves. The level of jasmonic acid in noninoculated leaves of pathogen-treated plants 72 hr after inoculation was approximately fourfold higher than in corresponding leaves of mock-inoculated plants. It appears from these experiments that the induction of plant defensin genes upon pathogen challenge coincides with a rise in endogenous jasmonic acid levels, both in inoculated and in noninoculated, systemic leaves.

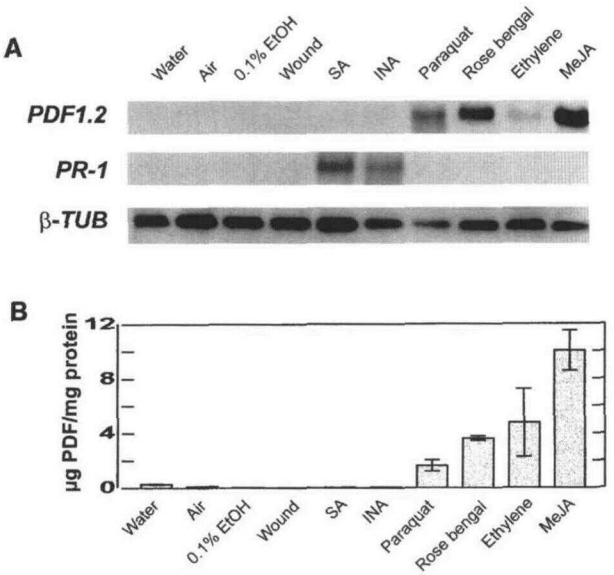

Figure 4. Induction of Plant Defensins and *PR-1* in Arabidopsis Leaves Chemically Treated or Wounded.

(A) RNA gel blot analysis of *PDF1.2*, *PR-1*, and β-tubulin (*β-TUB*) expression in leaves wounded or treated with the indicated chemicals. All analyzed samples represent 4 μg of total RNA.

(B) Plant defensin (PDF) content as determined by ELISA, using antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (± SE) of three independent determinations.

Arabidopsis leaves were inoculated with 5-μL drops (five drops per leaf) of water, SA (5 mM), INA (1 mg/mL), paraquat (25 μM), rose bengal (20 mM), methyl jasmonate (MeJA; 45 μM in 0.1% [v/v] ethanol), or 0.1% (v/v) ethanol (0.1% EtOH). Ethylene treatment was performed by placing plants in an air-tight chamber with an ethylene concentration of 20 ppm. Control plants (air) were incubated in an identical chamber without ethylene. Leaves were wounded by making incisions in the leaves with a scalpel. All of the leaf samples were collected 48 hr after initiation of the treatments. The experiment was repeated once with similar results.

Systemic Induction of Plant Defensins in Arabidopsis Follows an SA-Independent Pathway

Because SA and INA were unable to induce the synthesis of plant defensins, we further investigated the role of SA in the induction of plant defensins after fungal infection. Therefore, we measured plant defensin gene expression in Arabidopsis plants known to be genetically modified in the SA signaling pathway. Transgenic *nahG*-expressing Arabidopsis plants do not accumulate significant amounts of SA when infected by a pathogen due to the conversion of SA to catechol (Delaney et al., 1994; Lawton et al., 1995). The *npr1* (for nonexpresser of PR genes) mutant displays a blocked SA signaling pathway because it is unable to express PR genes when treated with SA or attacked by a pathogen (Cao et al., 1994). In contrast, the *cpr1* (for constitutive expresser of PR genes) mutant has constitutively elevated SA and PR protein levels even in the absence of any stress signal.

Plants of different Arabidopsis lines affected in the SA signaling pathway were either mock-inoculated with water or inoculated with a suspension of spores from *A. brassicicola*, and treated and nontreated (systemic) leaves were harvested after 72 hr. The expression of plant defensin genes was measured both by RNA gel blot analysis and ELISA, whereas expression of *PR-1* was measured only by RNA gel blot analysis. When wild-type (Col-0) plants were inoculated with *A. brassicicola*, expression of plant defensin genes and *PR-1* was induced in both pathogen-treated leaves and nontreated, systemic leaves when compared with that of the corresponding leaves of mock-inoculated plants (Figure 6). In *nahG*-expressing plants, the induction of *PR-1* upon infection was completely blocked both in pathogen-treated and nontreated, systemic leaves. In marked contrast, however, plant defensins were strongly induced in pathogen-treated

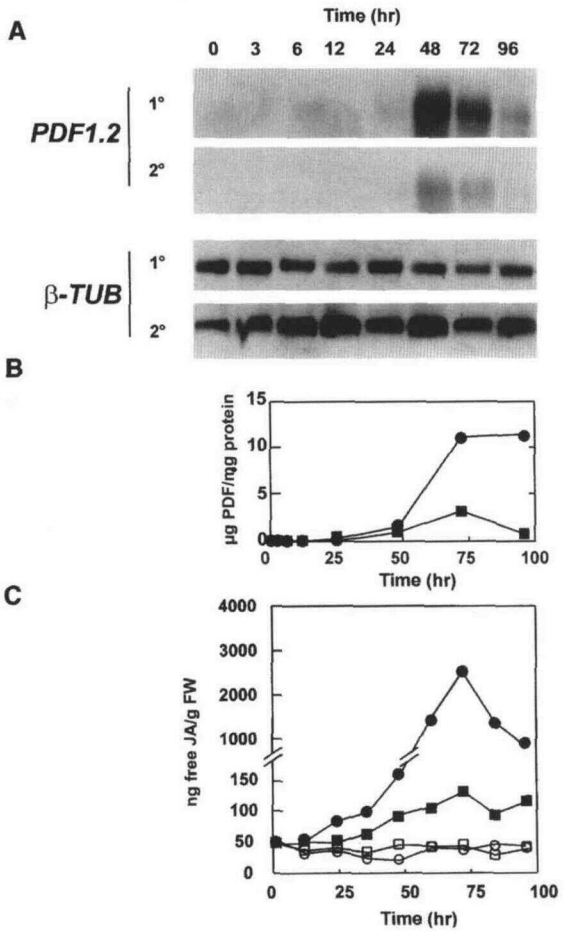

Figure 5. Accumulation of Plant Defensins and Jasmonic Acid in Arabidopsis after Fungal Infection.

(A) RNA gel blot analysis of *PDF1.2* and β-tubulin (*β-TUB*) expression in pathogen-treated leaves (1°) and nontreated, systemic leaves (2°) of infected plants. All of the analyzed samples represent 4 μg of total RNA.

(B) Plant defensin (PDF) content in pathogen-inoculated leaves (filled circles) and nontreated, systemic leaves (filled squares) of inoculated plants, as determined by ELISA, using antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (±SE) from three independent determinations.

(C) Endogenous jasmonic acid content in pathogen-inoculated leaves (filled circles), nontreated, systemic leaves of inoculated plants (filled squares), mock-inoculated leaves (open circles), and nontreated, systemic leaves of mock-inoculated plants (open squares).

Total RNA in **(A)** and proteins in **(B)** were isolated from pathogen-treated and nontreated, systemic Arabidopsis leaves collected 0, 3, 6, 12, 24, 48, 72, and 96 hr after inoculation with 5-μL drops (five drops per leaf) of *A. brassicicola* spores at 5 × 10^5 spores per mL. Jasmonic acid (JA) levels in **(C)**, expressed as nanograms of free jasmonic acid per gram fresh weight (FW), were determined by HPLC in extracts from treated and nontreated, systemic Arabidopsis leaves collected 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr after inoculation with 5-μL drops (five drops per leaf) of either *A. brassicicola* spores at 5 × 10^5 spores per mL or distilled water. Lesions were macroscopically apparent on the leaves ~36 hr after inoculation. Plants analyzed in **(C)** were from a different batch than those analyzed in **(A)** and **(B)**. All experiments were repeated once and showed similar trends.

nahG-expressing plants both locally, in pathogen-treated leaves, and systemically, in the nontreated leaves. The levels of plant defensins, measured by ELISA, in pathogen-treated and nontreated, systemic leaves of inoculated plants were consistently two- to threefold higher in *nahG*-expressing plants compared with wild-type plants.

A similar observation was made with the *npr1* mutant. In this mutant, pathogen-induced expression was fully suppressed for *PR-1* but not for the plant defensin genes. Finally, a constitutively enhanced level of *PR-1* expression, but not of plant defensin genes, was observed in mock-inoculated *cpr1* plants. When *cpr1* plants were challenged with *A. brassicicola*, no further accumulation of *PR-1* mRNA could be observed above the levels in mock-inoculated plants, whereas expression of plant defensin genes was clearly induced.

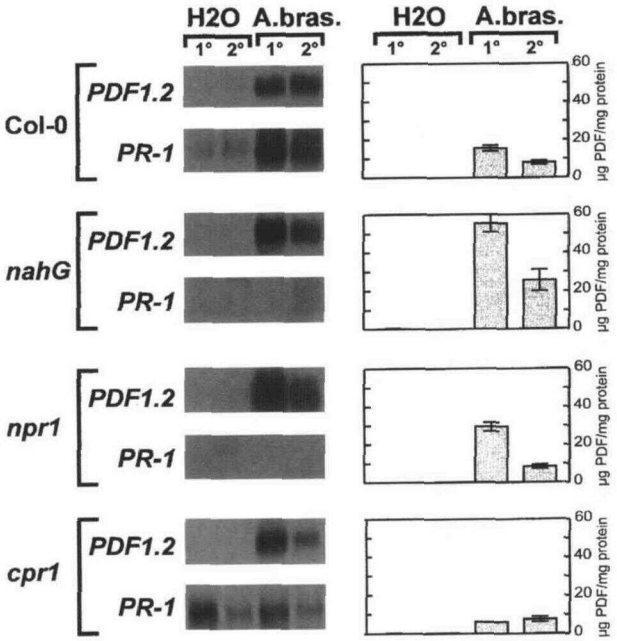

Figure 6. Induction of Plant Defensins and *PR-1* in Arabidopsis Wild Type (Col-0), in an SA-Deficient Arabidopsis Transformant (*nahG*), and in Arabidopsis Mutants (*npr1* and *cpr1*) Affected in the SA Signaling Pathway.

(Left) RNA gel blots showing *PDF1.2* and *PR-1* expression. The samples represent 4 μg of total RNA.

(Right) Plant defensin (PDF) contents as determined by ELISA, using antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (±SE) of three independent determinations.

Arabidopsis plants were inoculated with *A. brassicicola* (A.bras.) by applying 5-μL drops of a spore suspension (5 × 10^5 spores per mL) on four lower rosette leaves (five drops per leaf). Control plants were treated identically with 5-μL drops of water (H_2O). Pathogen-treated leaves (1°) and nontreated leaves of the same plants (2°) were collected 3 days after inoculation. Total RNA and proteins were extracted as described in Methods. The experiment was repeated twice with similar results.

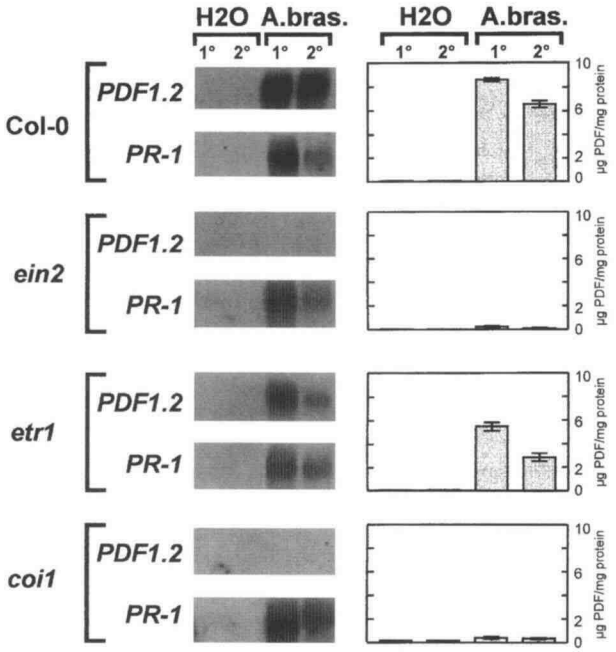

Figure 7. Induction of Plant Defensins and *PR-1* in Arabidopsis Wild Type (Col-0), in Arabidopsis Mutants (*ein2* and *etr1*) Affected in the Ethylene Response Pathway, and in a Mutant (*coi1*) Affected in the Jasmonate Response Pathway.

(Left) RNA gel blots showing *PDF1.2* and *PR-1* expression. The samples represent 4 µg of total RNA.

(Right) Plant defensin (PDF) contents as determined by ELISA, using antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (±SE) of three independent determinations.

Arabidopsis plants were inoculated with *A. brassicicola* (A.bras.) by applying 5-µL drops of a spore suspension (5 × 10^5 spores per mL) on four lower rosette leaves (five drops per leaf). Control plants were treated identically with 5-µL drops of water (H_2O). Pathogen-treated leaves (1°) and nontreated leaves of the same plants (2°) were collected 3 days after inoculation. Total RNA and proteins were extracted as described in Methods. The experiment was repeated twice with similar results.

Pathway for Induction of Plant Defensins Requires Components of the Ethylene and Jasmonate Response Pathways

As shown above, plant defensins accumulate in leaves of Arabidopsis plants after application of exogenous ethylene or methyl jasmonate. To clarify the role of these two plant hormones in the induction of plant defensins, we investigated expression of plant defensin genes and *PR-1* in the ethylene-insensitive mutants *ein2* and *etr1-3* (Guzmán and Ecker, 1990; Chang et al., 1993) as well as in the coronatine- and methyl jasmonate–insensitive mutant *coi1* (Feys et al., 1994).

In response to fungal infection, *PR-1* mRNA accumulated in infected and systemic leaves in all three mutants at levels similar to those observed in wild-type plants (Figure 7). Thus, it appears that ethylene insensitivity and jasmonate insensitivity do not influence significantly the induction of *PR-1*. In contrast, the induction of plant defensins was markedly affected when the ethylene-insensitive or jasmonate-insensitive mutants were infected with *A. brassicicola*. The induction of plant defensins appeared to be almost totally blocked in *ein2* and *coi1* plants. The levels of plant defensins in pathogen-treated and nontreated, systemic leaves of *ein2* and *coi1* plants after inoculation with *A. brassicicola* were at least 30-fold lower than were the levels in the corresponding leaf samples of similarly pathogen-treated wild-type plants. The block in pathogen-induced expression of plant defensin genes in *ein2* and *coi1* plants occurred at the transcriptional level because no plant defensin mRNA was detected in leaves of pathogen-treated plants.

In *etr1-3* plants, expression of plant defensin genes was enhanced in the pathogen-treated leaves to levels similar to those found in pathogen-treated leaves of wild-type plants. However, in nontreated, systemic leaves of *etr1-3* plants, accumulation of plant defensins was increased to a level that was threefold lower than in the systemic leaves of wild-type plants. This reduction was consistent throughout three independent experiments, but the extent of reduction was variable (five- to twofold lower relative to wild-type plants).

Plant Defensins and Jasmonic Acid Levels Are Elevated in the Lesion-Mimic Mutant *acd2*

The Arabidopsis *acd2* mutant spontaneously develops lesions similar to those developed by wild-type plants undergoing a hypersensitive response when challenged with avirulent bacterial pathogens (Greenberg et al., 1994). Because this mutant has previously been shown to accumulate high levels of PR gene transcripts in both asymptomatic and necrotic leaves, it was considered worthwhile to assess plant defensin gene expression in *acd2* plants. Healthy asymptomatic upper rosette leaves and lower rosette leaves displaying necrotic lesions were harvested separately from 5-week-old *acd2* plants, as were healthy upper and lower rosette leaves from wild-type (Col-0) plants grown under the same conditions. As measured by ELISA, *acd2* plants accumulated very high levels of plant defensins, estimated to constitute ~5 and 10% of total soluble proteins in asymptomatic leaves and leaves with necrotic lesions, respectively (Figure 8B). RNA gel blot analysis showed that transcript levels of both plant defensins and *PR-1* in necrotic as well as in asymptomatic leaves of *acd2* were strongly elevated compared with those in wild-type plants (Figure 8A).

The endogenous jasmonic acid content of *acd2* plants was also determined and found to be ninefold higher in leaves with necrotic lesions and threefold higher in asymptomatic leaves versus the levels in the corresponding leaves from wild-type (Col-0) plants.

DISCUSSION

Analysis of the expression of two Arabidopsis plant defensin genes identified via their EST sequences revealed that they

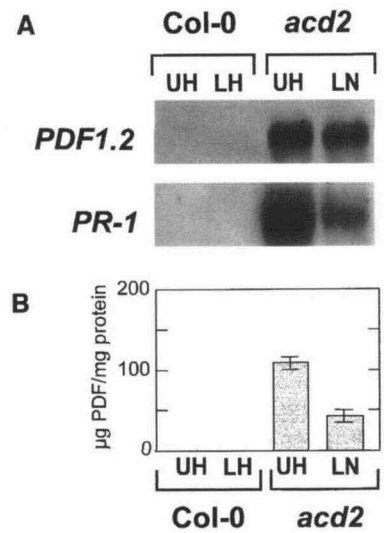

Figure 8. Induction of Plant Defensins and *PR-1* in Arabidopsis Wild Type (Col-0) and in an Arabidopsis Lesion-Mimic Mutant (*acd2*).

(A) RNA gel blots showing *PDF1.2* and *PR-1* expression. All of the analyzed samples represent 4 µg of total RNA.

(B) Plant defensin (PDF) content as determined by ELISA, using antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (± SE) of three independent determinations.

Total RNA and proteins were isolated from healthy asymptomatic upper rosette leaves (UH) and lower rosette leaves displaying necrotic lesions (LN) collected from 5-week-old *acd2* plants, as well as from healthy upper rosette (UH) and lower rosette leaves (LH) from control plants (Col-0) grown under identical conditions. The experiment was repeated twice with similar results.

are differentially expressed, and only one of these two genes, designated *PDF1.2*, appears to be pathogen inducible. Although only two homologous plant defensin EST sequences have been identified to date, it is possible that other plant defensin genes may be present and possibly expressed in Arabidopsis. Gel blot analysis of Arabidopsis genomic DNA digested with four independent restriction enzymes and using a cDNA clone for *Rs-AFP2* as hybridization probe revealed three to five bands, depending on the enzyme used (data not shown). Currently, the expression pattern of other putative plant defensin genes during pathogenesis remains unknown and requires a careful examination of the gene family.

Before this study, three Arabidopsis genes have been identified, namely, *PR-1*, *PR-2* (encoding a β-1,3-glucanase), and *PR-5* (encoding an osmotin-like protein). They are coordinately and systemically induced when infected with a pathogen (Dempsey et al., 1993; Uknes et al., 1993; Mauch-Mani and Slusarenko, 1994). These genes are all highly induced by the exogenous application of SA or INA, a synthetic compound that appears to mimic the action of SA (Uknes et al., 1992; Cao et al., 1994). In this work, we found that the expression of plant defensin genes, including at least *PDF1.2*, is also induced systemically upon pathogen infection, but this induction follows a response pathway that is clearly different from that followed by *PR-1*. This finding is based on several lines of evidence.

First, Arabidopsis plant defensins were not induced when either SA or INA was applied externally. In contrast, treatments with either methyl jasmonate, ethylene, or paraquat and rose bengal—compounds that generate reactive oxygen species—caused the accumulation of plant defensin transcripts but not *PR-1* transcripts. Second, the pathogen-induced systemic expression of plant defensin genes was not reduced in transgenic *nahG*-expressing plants that are unable to accumulate both SA and PR-1 when infected (Delaney et al., 1994; Lawton et al., 1995). The same holds true for the *npr1* mutant that is known to be blocked in the response pathway leading from SA to activation of PR genes (Cao et al., 1994). Third, plant defensin genes were not constitutively expressed in the *cpr1* mutant, which displays constitutively elevated levels of endogenous SA as well as of *PR-1*, *PR-2*, and *PR-5* transcripts (Bowling et al., 1994). Fourth, analysis of the *etr1-3* (previously called *ein1-1*), *ein2*, and *coi1* mutants of Arabidopsis demonstrated that the mechanism leading to induction of plant defensins in response to fungal challenge appears to involve components from or shared by the ethylene and jasmonate response pathways, whereas these components are not required for *PR-1* induction. Therefore, these results indicate that two separate classes of antifungal proteins (PR-1 and plant defensins) can be induced both locally and systemically by distinct signaling processes.

The *ein2* Arabidopsis mutant, originally identified by a lack of morphological response when grown in the presence of ethylene (Guzmán and Ecker, 1990), is virtually blocked in its pathogen-induced expression of plant defensin genes both in pathogen-treated leaves and in nontreated, systemic leaves. In contrast, the *etr1-3* mutant, which like *ein2* does not show an altered growth response upon exposure to ethylene (Bleecker et al., 1988; Chang et al., 1993), has a normal plant defensin response in infected leaves but exhibits reduced but not abolished plant defensin gene expression in nontreated leaves of infected plants. The *coi1* mutant is known to be less sensitive than are wild-type plants to inhibition of root growth when treated with methyl jasmonate or coronatine, a bacterial phytotoxin acting as a jasmonate analog (Feys et al., 1994). This mutant showed an almost completely blocked pathogen-induced plant defensin response both in the pathogen-treated and nontreated, systemic leaves. Of the genes affected in these three mutants, only *ETR1* has been identified. *ETR1* encodes a protein resembling bacterial two-component histidine kinase sensors, and genetic and biochemical evidence indicates that ETR1 is an ethylene receptor (Schaller and Bleecker, 1995). The *EIN2* gene product acts downstream of ETR1 in the ethylene response pathway (Ecker, 1995). COI1 has not been characterized to date but is believed to be involved in signal transduction initiated by jasmonates (Feys et al., 1994).

Interestingly, *ein2* plants have previously been found to display decreased chlorotic lesion formation relative to wild-type plants when infiltrated with virulent strains of the bacterium

Pseudomonas syringae pv *tomato* (Bent et al., 1992). Mutants carrying the *etr1-3* mutation responded like wild-type plants. Although *ein2* plants showed decreased disease symptoms relative to wild-type and *etr1-3* plants, *P. s. tomato* multiplied equally well in these three genotypes. The reduced disease symptoms in leaves of the *ein2* mutant infiltrated with virulent bacteria may result from the lack of local accumulation of a defense compound, for example, a phytoalexin that is phytotoxic on one hand and ineffective against the bacterium on the other. However, in our case the degree of infection and disease symptoms in *ein2* mutants challenged with *A. brassicicola* was not reduced but consistently enhanced compared with wild-type plants (B.P.H.J. Thomma and I.A.M.A. Penninckx, unpublished observations).

Although the requirement of EIN2 in the plant defensin response is unequivocal, the involvement of ETR1 and of ethylene itself is unclear. Our observation that the local plant defensin response is unaffected in the *etr1-3* mutant, a mutant blocked at the level of the ethylene receptor, seems to argue against direct involvement of ETR1 and ethylene, at least in the local response. However, the results obtained with the *etr1-3* mutant should be interpreted with care, considering that this mutant is not fully blocked in its response to ethylene (J. Ecker, personal communication). Hence, the *etr1-3* allele may be partially effective in blocking the systemic plant defensin response but not the more intense local response. Similar observations have been made with transgenic *nahG*-expressing tobacco plants in which the induction of *PR-1* was blocked in noninoculated, systemic leaves but not in inoculated leaves (Vernooij et al., 1994).

A model for two separate pathways leading to induction of genes upon pathogen stress is presented in Figure 9. In this model, the hypersensitive response is positioned above the bifurcation point, because *acd2* mutants developing spontaneous hypersensitive response–like lesions (Greenberg et al., 1994) were found to contain enhanced transcript levels of both plant defensins and *PR-1*. Greenberg et al. (1994) showed previously that *acd2* mutants accumulate more SA than do wild-type plants. We have now shown that *acd2* plants also produce more jasmonic acid. The pathway leading to PR gene expression via SA involves the unknown signal transduction components NPR1 and CPR1, whereas the pathway leading to plant defensin gene expression requires EIN2 and COI1. Several lines of indirect evidence point to a pivotal role of jasmonic acid in the latter pathway. First, we have shown that endogenous jasmonic acid and plant defensin levels rise simultaneously when Arabidopsis is challenged with *A. brassicicola*, both in inoculated and noninoculated, systemic leaves. Second, exogenous application of methyl jasmonate leads to enhanced plant defensin accumulation. Third, the *coi1* mutant, which is not responsive to methyl jasmonate, does not accumulate plant defensins in response to challenge by *A. brassicicola*. However, direct evidence for the requirement of endogenous jasmonic acid to activate the plant defensin response is lacking at this time. The fact that *ein2* blocks plant defensin induction suggests, but certainly does not prove, the involvement of ethylene as well. Assuming that ethylene is involved, it remains to be demonstrated whether it acts upstream, downstream, or in concert with jasmonic acid. In this respect, it is interesting that jasmonic acid and ethylene have a synergistic effect on the induction of an osmotin gene in tobacco (Xu et al., 1994).

There may be some degree of "cross-talk" between both the SA-dependent and SA-independent response pathways. One indication of cross-talk stems from the observation that plant defensin accumulation in pathogen-stressed *nahG*-expressing plants was consistently at least twofold higher than that in wild-type plants. Thus, it appears that inhibition of SA accumulation results in enhanced activation of the SA-independent pathway. In this respect, it is worth noting that acetylsalicylic acid was shown to inhibit jasmonate biosynthesis (Peña-Cortes et al., 1993), whereas SA was found to inhibit the induction of proteinase inhibitors by jasmonic acid (Doares et al., 1995).

The existence of multiple defense response pathways in Arabidopsis has previously been inferred from the observation that two different hypersensitive response–provoking bacterial pathogens that differ only in a single avirulence gene each activate different sets of genes (Reuber and Ausubel, 1996). It has also recently been shown that exposure of Arabidopsis to ozone coactivates both *PR-1* in an SA-dependent manner and a phenylalanine ammonia-lyase gene in an SA-independent manner (Sharma et al., 1996). The latter response has been suggested but not proven to be mediated by jasmonic

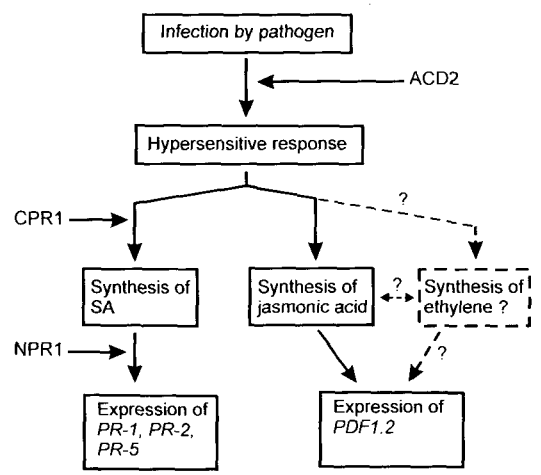

Figure 9. Proposed Model for Local Induction of Defense-Related Genes via Two Separate Pathways.

The relative placement of CPR1 and NPR1 in the SA-dependent pathway (left branch) is according to Bowling et al. (1994). The exact position of EIN2 and COI1 in the SA-independent pathway has yet to be determined. The involvement of ethylene in the SA-independent pathway is speculative at present, and it is also not known whether ethylene and jasmonate act in sequence or in parallel.

acid (Sharma et al., 1996). Ozone is a gas that generates reactive oxygen species when absorbed by plant tissues (Kanofsky and Sima, 1995). Exposure of plants to ozone results in accumulation of SA (Sharma et al., 1996) as well as enhanced production of ethylene (Mehlhorn and Wellburn, 1987). Because the hypersensitive response of plants to incompatible pathogens also involves generation of reactive oxygen species (reviewed in Mehdy, 1994), it is not surprising that the response pathways to pathogens and ozone overlap substantially. Reactive oxygen species generated during the hypersensitive response are likely to play a key role as early signal molecules triggering both the SA-dependent and SA-independent pathogen response pathways.

Other defense-related genes may be activated coordinately with plant defensins via the SA-independent pathway depicted above. A first likely candidate is *Hel*, a hevein-like (PR-4–like) gene that is induced both locally and systemically upon viral infection (Potter et al., 1993). *Hel* is strongly induced by ethylene but only weakly by SA, whereas *PR-1*, *PR-2*, and *PR-5* are not ethylene inducible but are strongly SA inducible (Potter et al., 1993). A second candidate is the thionin gene *Thi2.1*, which is induced by fungal infection and methyl jasmonate treatment but not by treatment with SA (Epple et al., 1995). A third possible candidate is the basic chitinase gene *CHIT-B*, which is induced by ethylene treatment of wild-type plants but not of *ein2* or *etr1-1* mutants (Chen and Bleecker, 1995). It was observed that the induction of *CHIT-B* in virus-infected leaves of Arabidopsis followed different kinetics relative to the induction of *PR-1*, *PR-2*, and *PR-5* (Dempsey et al., 1993).

There is also evidence for the existence of two separate pathways for pathogen-inducible responses in tobacco. The first pathway leads to induction of acidic PR genes such as *PR-1*, *PR-2*, *PR-3* (encoding acidic chitinase), *PR-4*, and *PR-5*, whereas the second pathway results in induced expression of the basic β-1,3-glucanase and basic chitinase genes. The first group of genes is strongly activated by SA; the second group responds to ethylene (Meins et al., 1991). Interestingly, transgenic tobacco plants expressing a gene encoding the A1 subunit of cholera toxin, a G protein inhibitor, showed constitutive expression of the acidic PR protein genes but not of the basic β-1,3-glucanase and basic chitinase genes (Beffa et al., 1995). The acidic PR protein genes are induced both locally and systemically upon challenge with tobacco mosaic virus (Brederode et al., 1991; Ward et al., 1991). The basic β-1,3-glucanase and basic chitinase genes, on the other hand, are also strongly induced in the inoculated leaves, but there is contradictory evidence of their systemic induction. Based on RNA gel blot analysis, no significantly enhanced transcript levels of these genes could be detected in uninoculated leaves of tobacco mosaic virus–infected plants (Brederode et al., 1991; Ward et al., 1991), whereas the corresponding proteins were found to accumulate in such leaves based on protein gel blot analyses (Heitz et al., 1994).

We speculate that pathogen-induced expression of the basic β-1,3-glucanase and basic chitinase genes in tobacco follows a pathway equivalent to that leading to induced plant defensin gene expression in Arabidopsis. One apparent difference is that challenge of Arabidopsis with a pathogen results in enhanced systemic accumulation of both plant defensin transcripts and proteins. It should be taken into account that Arabidopsis is much smaller than is tobacco and hence that systemic responses in the former are measured over much shorter distances. Another obvious difference between basic β-1,3-glucanase and basic chitinase expression in tobacco on the one hand and plant defensin gene expression in Arabidopsis on the other is that the tobacco genes are wound inducible (Brederode et al., 1991), whereas plant defensin genes are not.

Our observation that plant defensins in Arabidopsis leaves are induced when treated with methyl jasmonate but not when wounded is somewhat puzzling, considering that wounding of Arabidopsis leaves results in enhanced jasmonic levels (A. Buchala, unpublished observations). Another example of a jasmonate-inducible gene that is not responsive to wounding is the papain inhibitor gene from tomato leaves (Bolter, 1993). It has previously been suggested that jasmonic acid synthesis can take place in distinct cellular compartments, each of which can be activated by distinct signals to trigger distinct responses (Harms et al., 1995). On the other hand, wounding may, in addition to boosting jasmonic acid levels, result in the release of other signals that may act in concert to activate particular genes and downregulate others. In this concept, the lack of induction of plant defensin genes to wounding may result from inhibition of expression due to additional wound signals.

We have demonstrated that a pathogen-induced protein cross-reacting with antibodies raised against a PDF1.2 analog from radish (Rs-AFP1) possesses strong antifungal activity in vitro. When infected by a fungus, this protein accumulates to high levels: up to 2 and 1% of total proteins in inoculated and nontreated, systemic leaves, respectively. We have previously shown that transgenic tobacco plants producing a PDF1.2 analog from radish (Rs-AFP2) at ~0.25% of total soluble proteins are more resistant to infection by *A. longipes* than are untransformed plants (Terras et al., 1995). Based on these observations, it is likely that plant defensins have a role to play in host defense. There is now compelling evidence that an SA-dependent pathway is important for mounting resistance in Arabidopsis to some pathogens, including *P. s. tomato* and *Peronospora parasitica*. Indeed, *nahG*-expressing Arabidopsis plants were found to be considerably more sensitive to these pathogens compared with wild-type plants (Delaney et al., 1994) and, unlike wild-type plants, were unable to mount SAR (Lawton et al., 1995). However, it is conceivable that the SA-independent pathway leading to activation of plant defensin genes, and possibly other defense-related genes as well, could also contribute to resistance. Two Arabidopsis mutants, *ein2* and *coi1*, that are apparently blocked in the SA-independent pathway will be particularly useful to verify this hypothesis. Our preliminary results indicate that *ein2* plants are highly sensitive to decay by the soft rot fungus *Botrytis cinerea*, whereas wild-type plants as well as *nahG*-expressing plants almost fully survive challenge by this fungus (B.P.H.J. Thomma, unpublished results).

METHODS

Biological Material

The transgenic *Arabidopsis thaliana* line containing the *nahG* gene (Delaney et al., 1994) was obtained from J. Ryals (Ciba Geigy, Research Triangle Park, NC). The mutants *npr1* (Cao et al., 1994) and *cpr1* (Bowling et al., 1994) were provided by X. Dong (Duke University, Durham, NC). The ethylene response mutants *ein2* (Guzmán and Ecker, 1990) and *etr1-3* (Bleecker et al.,1988; Chang et al., 1993) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH; accession numbers CS3071 and CS3070, respectively), and the lesion mimic mutant *acd2* (Greenberg et al., 1994) was provided by F. Ausubel (Massachusetts General Hospital, Boston, MA). The jasmonate response mutant *coi1* (Feys et al., 1994) was obtained from J. Turner (University of East Anglia, Norwich, UK). Because this mutation is recessive and causes male sterility, *coi1* mutants were identified in F_2 plants grown from seed from selfed *COI1/coi1* hemizygous plants. Therefore, the F_2 population was subjected to different treatments, as indicated below. Leaves from each individual were collected separately, and the plants were then grown until seed set. Individuals that did not form siliques were identified as having the *coi1/coi1* genotype. All of the mutant and transgenic lines listed above are derived from the Columbia (Col-0) ecotype. Growth and spore harvesting of the fungus *Alternaria brassicicola* (MUCL 20297; Mycothèque Université Catholique de Louvain, Louvain-la-Neuve, Belgium) were done as described previously (Broekaert et al., 1990).

Plant Growth Conditions, Chemical Application, and Inoculation

Arabidopsis seed were sown on flower potting compost containing a macronutrient supplement (Asef, Didam, The Netherlands) in Petri dishes. Seed were vernalized for 2 days at 4°C after sowing. After 5 days of incubation in a growth chamber (20°C daytime temperature and 18°C nighttime temperature, with a 12-hr photoperiod at a photon flux density of 100 $\mu E\ m^{-2}\ sec^{-1}$), seedlings were transferred to pots (5 × 4 × 4 cm) containing potting compost supplemented with macronutrients and grown under the same conditions as above. Irrigation was with tap water via the trays carrying the pots. Plants were 4 weeks old when treated. Leaves were wounded by crushing with forceps with ribbed tips or by making incisions into the limb with a scalpel, taking care to leave the midvein intact.

Paraquat (25 μM), rose bengal (20 mM), methyl jasmonate (45 μM in 0.1% [v/v] ethanol), 0.1% (v/v) ethanol, sodium salicylate (5 mM), and 2,6-dichloroisonicotinic acid (INA; 1 mg/mL of a 25% active ingredient in a wettable powder, provided by H. Kessmann, Novartis, Basel, Switzerland) were applied at the concentrations indicated as 5-μL droplets on leaves (5 drops per leaf). The stock solution of methyl jasmonate was 45 mM in ethanol. Ethylene treatment was performed by placing pots in an air-tight translucent chamber in which gaseous ethylene was injected via a silicon rubber septum. The ethylene concentration in the chamber (20 ppm) was verified by gas chromatography. Control plants for the ethylene experiment were placed in an identical chamber without ethylene.

Inoculation with *A. brassicicola* was done by applying 5-μL drops of a spore suspension (density of 5 × 10^5 spores per mL in distilled water) on the cotyledons and on the four oldest rosette leaves (five drops per leaf). Control plants were treated identically with water droplets. The plants with drops of spore suspension or water were placed randomly (if different genotypes were treated simultaneously) in a propagator flat with a clear polystyrene lid and kept at high humidity for 2 days to stimulate infection by hyphal germlings. Thereafter, lids were taken off and the plants were incubated further until the harvesting of leaf material. The treated leaves (cotyledons and rosette leaf numbers 1 to 4) were collected separately from the nontreated leaves (rosette leaf numbers 5 to 8) of the same plants. The isolate of *A. brassicicola* and inoculation conditions used here caused limited brown necrotic lesions under the drops of spore suspension within 36 hr of inoculation, and these lesions failed to spread further. Microscopic inspection indicated that although spore germination and attempted penetration had occurred on the leaf surface of Arabidopsis Col-0 plants, there was no evidence of substantial hyphal growth or sporulation either within or on the leaf.

RNA Gel Blot Analysis

RNA was extracted by the phenol-LiCl method according to Eggermont et al. (1996), from tissues frozen in liquid nitrogen and stored at −80°C. RNA samples were loaded at 4 μg per lane on a formaldehyde–agarose gel and blotted onto a positively charged nylon membrane (Boehringer Mannheim) via capillary transfer with 20 × SSC (1 × SSC is 0.15 mM NaCl, 0.015 mM sodium citrate; Sambrook et al., 1989). To verify equal loading and transfer of RNA, we supplemented the loading buffer with 50 μg/mL ethidium bromide, allowing visualization of RNA in the gels and on the blots when illuminated with UV light. RNA was cross-linked on the blots by UV illumination of both sides for 5 min each. Blots were prehybridized, hybridized with digoxigenin-labeled antisense RNA probes, and developed by immunochemiluminescence, as described previously (Terras et al., 1995). Digoxigenin-labeled probes were made by run-off transcription, using the Dig RNA labeling kit of Boehringer Mannheim. The *PDF1.2* probe was synthesized using SP6 RNA polymerase and the EcoRI-linearized plasmid pZL1 (Bethesda Research Laboratory Life Technologies, Gaithersburg, MD) containing the expressed sequence tag (EST) with GenBank accession number T04323 (obtained from the Arabidopsis Biological Resource Center). The probe for the tubulin β-1 chain gene was synthesized using T7 RNA polymerase and the EcoRI-linearized plasmid pBluescript II SK− (Stratagene, La Jolla, CA) containing the EST with GenBank accession number Z26191 (obtained from D. Roby, Institut National de la Recherche Agronomique, Castanet-Tolosan, France). The pathogenesis-related *PR-1* probe was synthesized using SP6 RNA polymerase and the EcoRI-linearized plasmid pFRG13b. Plasmid pFRG13b was constructed by subcloning the EcoRI-XhoI insert from a pBluescript II SK− plasmid carrying the Arabidopsis *PR-1* cDNA (obtained from L. Friedrich, Ciba Geigy) into the EcoRI and SalI sites of plasmid pSPT18 (Boehringer Mannheim). Samples analyzed with different probes were run on replicate gels that were developed separately.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA (1 μg in 100 mM sodium acetate, 5 mM $MgSO_4$, pH 5.0) was treated with six units of RNase-free Dnase I (Boehringer Mannheim) at 37°C for 5 min, after which the DNase was inactivated by heating at 95°C for 5 min. The RNA was precipitated overnight in the presence of 0.2 M sodium acetate and 66% (v/v) ethanol, collected by centrifugation at 10,000*g* for 10 min, washed twice with 70% (v/v) ethanol, and finally dissolved in RNase-free water. The reverse transcriptase–polymerase chain reactions (RT-PCRs) were performed with

1 μg of DNase-treated total RNA with 10 units of avian myeloblastosis virus reverse transcriptase (Pharmacia, Uppsala, Sweden) for 60 min at 52°C. The RT-PCRs were performed with a homopolymeric deoxythymidine oligonucleotide (20-mer) and terminated by the addition of Na-EDTA to a final concentration of 15 mM. A fraction (one-thirtieth) of the reverse transcription reaction solution was used as a template in a 50-μL PCR performed with 2.5 units of Taq polymerase (Appligene, Pleasanton, CA), according to Sambrook et al. (1989).

PCR was run for 30 cycles with annealing temperatures of 55, 65, and 65°C for amplification with primer pairs specific for *PDF1.1*, *PDF1.2*, and *ACTIN-1*, respectively. Primers used for amplification of *PDF1.1* were OWB260 (sense, 5′-GAGAGAAAGCTTGTTGTGCGAGAGGCCAAGTGGG-3′) and OWB259 (antisense, 5′-GAGAGAGGATCCTGCAAGATCCATGTCGTGCTTTC-3′); those for amplification of *PDF1.2* were OWB240 (sense, 5′-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3′) and OWB241 (antisense, 5′-AATCCATGGAATACACACGATTTAGCACC-3′); and those for amplification of *ACTIN-1* were OWB270 (sense, 5′-GGCGATGAAGCTCAATCCAAACG-3′) and OWB271 (antisense, 5′-GGTCACGACCAGCAAGATCAAGACG-3′).

Purification of Plant Defensins from *A. brassicicola*-Infected Arabidopsis Leaves

Leaves of 5-week-old Arabidopsis plants were inoculated with 5-μL drops of distilled water (control) or a *A. brassicicola* spore suspension (5×10^5 spores per mL in water) and collected after 3 days of incubation in a moist propagator flat with a clear polystyrene lid. Extracts were prepared from 20 g of either water-treated or inoculated leaves and subjected to the purification procedure, exactly as previously described in Terras et al. (1995). Protein analysis, in vitro antifungal activity analysis, and SDS-PAGE on precast PhastGel High Density gels (Pharmacia) were performed as previously described (Terras et al., 1995). Before SDS-PAGE analysis, protein samples were reduced with DTT and *S*-pyridylethylated, as given in Terras et al. (1992). Immunoblotting of proteins, separated on a 15% acrylamide–SDS–polyacrylamide gel, was done as described in Terras et al. (1995).

ELISA

Proteins were isolated from frozen leaf material in extraction buffer (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, and 1.5% [w/v] polyvinylpolypyrrolidone, pH 7). Protein concentrations were determined in the crude extracts, according to Bradford (1976), using BSA as a standard. After heat treatment (10 min, 80°C) of the extract, the heat-stable soluble protein fraction was analyzed in a competition ELISA.

ELISA microtiter plates (Greiner Labortechnik, Frickenhausen, Germany) were coated with 100 ng/mL Rs-AFP2 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 2 hr at 37°C. The uncoated sites were blocked with 3% (w/v) cold fish skin gelatin (Sigma) in PBS for 2 hr at 37°C. Affinity-purified primary antibodies were diluted 50-fold in 0.3% (w/v) gelatin in PBS, containing 0.05% (v/v) Tween 20, and applied to the wells simultaneously with equal volumes (50 μL) of the samples diluted in the sample buffer. The plates were incubated for 1 hr at 37°C. After several washes with PBS containing 0.1% (v/v) Tween 20, the plate wells were filled (100 μL per well) with secondary antibodies (goat anti–rabbit antibodies coupled to alkaline phosphatase; Sigma) diluted 1000-fold in 0.3% (w/v) gelatin in PBS containing 0.05% (v/v) Tween 20. The plates were incubated for 1 hr at 37°C. Alkaline phosphatase activity was measured after 30 to 60 min of incubation at 37°C by using the substrate 4-nitrophenyl phosphate (Janssen Chimica, Beerse, Belgium) at a concentration of 1 mg/mL in substrate buffer (20 mM Na_2CO_3, 35 mM $NaHCO_3$, 5 mM $MgCl_2$, pH 9.6) by absorbance measurement at 405 nm. Protein samples were applied in a fourfold dilution series prepared in triplicate, and the plant defensin concentration was measured relative to a twofold dilution series of purified Rs-AFP1 (Terras et al., 1992), which was used as a standard.

Affinity purification of anti–Rs-AFP1 antiserum was as follows. An antigen affinity column was prepared by mixing equal volumes of 20 mg/mL purified Rs-AFP1 in 100 mM 3-*N*-morpholinopropanesulfonic acid buffer (pH 7) with Affi-Gel 10 matrix (Bio-Rad) equilibrated in water. The slurry was incubated overnight at 4°C with continuous gentle agitation. After blocking the unreacted sites of the matrix by the addition of 0.025 volumes of 1 M ethanolamine, pH 8, we subsequently washed the column with 10 mM Tris, pH 7.5, 100 mM glycine, pH 2.5, 10 mM Tris, pH 8.8, and 100 mM triethylamine, pH 11.5, and equilibrated it with 10 mM Tris, pH 7.5. The rabbit anti–Rs-AFP1 antiserum (Terras et al., 1995) was diluted twofold in ImmunoPure Gentle Ag/Ab binding buffer (Pierce Chemical Company, Rockford, IL) and passed several times over the affinity column. After washing the column with 15 volumes of ImmunoPure Gentle Ag/Ab binding buffer, we eluted purified anti–Rs-AFP1 antibodies with ImmunoPure Gentle Ag/Ab elution buffer (Pierce Chemical Company). The elution of bound antibodies was monitored by measuring the absorbance at 280 nm. The eluted fraction was dialyzed overnight against PBS.

Quantitative Analysis of Jasmonic Acid

The method for extraction of jasmonic acid is based on the one described by Gundlach et al. (1992). Arabidopsis leaves (~0.5 g) were frozen and ground in liquid nitrogen. The powder thus obtained was suspended in ethanol (5 mL) containing 500 ng of 9,10-dihydrojasmonic acid as an internal standard. The supernatant obtained after centrifugation (10 min at 4000*g*) was evaporated to dryness under reduced pressure at 40°C and subsequently resuspended in water (5 mL). After acidification to pH ~3 by the addition of concentrated HCl (0.3 mL), the solution was extracted with chloroform (2 × 5 mL). The organic phase was dried by the addition of anhydrous Na_2SO_4, decanted, and evaporated to dryness. The residue was dissolved in methanol (0.1 mL), and an ethereal solution of diazomethane (2 mL) was added. After at least 3 hr, the solution was evaporated to dryness under reduced pressure at room temperature. The residue was then dissolved in *n*-hexane (1 mL) and applied to a silica column (3 mL, Bakerbond spe; J.T. Baker Inc., Phillipsburg, NJ). The column was washed with *n*-hexane (7 mL), and the jasmonate-containing fraction was eluted with *n*-hexane-diethyl ether (1:2 [v/v], 5 mL). The solution was evaporated to dryness under reduced pressure at room temperature and redissolved in *n*-hexane (20 μL) for analysis by gas chromatography mass spectroscopy. Gas chromatography mass spectroscopy separations were performed on a capillary column (25 m × 0.2 mm) of BP-225 (Scientific Glass Engineering, Ringwood, Vic, Australia) fitted to a Hewlett Packard 5980 gas chromatograph (Palo Alto, CA) coupled to a Hewlett Packard 5970 mass specific detector. The methyl esters of jasmonic acid and 9,10-dihydrojasmonic acid were detected and quantitated by selective ion monitoring (electron impact of 70 eV) at m/z 224 and 156, respectively. The detection limit was found to be ~10 ng of jasmonate per gram fresh weight of tissue.

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