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cDNA-AFLP Reveals a Striking Overlap in Race-Specific Resistance and Wound Response Gene Expression Profiles

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The tomato *Cf-9* gene confers resistance to races of the fungal pathogen *Cladosporium fulvum* expressing the *Avr9* gene. cDNA amplified fragment length polymorphism analysis was used to display transcripts whose expression is rapidly altered during the Avr9- and Cf-9–mediated defense response in tobacco cell cultures. Diphenyleneiodonium was used to abolish the production of active oxygen species during gene induction. Of 30,000 fragments inspected, 290 showed altered abundance, of which 263 were induced independently of active oxygen species. cDNA clones were obtained for 13 *ACRE* (for *Avr9*/*Cf-9* rapidly elicited) genes. *ACRE* gene induction occurred in the presence of cycloheximide. Avr9 induced *ACRE* gene expression in leaves. Surprisingly, *ACRE* genes were also rapidly but transiently induced in leaves in response to other stresses. The amino acid sequences of some ACRE proteins are homologous to sequences of known proteins such as ethylene response element binding protein transcription factors, the N resistance protein, a calcium binding protein, 13-lipoxygenase, and a RING-H2 zinc finger protein. Rapid induction of *ACRE* genes suggests that they play a pivotal role during plant defense responses.

INTRODUCTION

Disease resistance in plants often involves recognition of invading pathogens followed by activation of a defense response. Such incompatible interactions are dependent on the presence of a resistance (*R*) gene in the host and an avirulence (*Avr*) gene in the pathogen (Flor, 1971; Keen, 1990). Many plant *R* genes have been identified. Their products have motifs consistent with potential roles in pathogen detection and subsequent signal transduction (Bent, 1996). However, the signal transduction activated by *R* gene products is still poorly understood.

Cladosporium fulvum is a biotrophic fungus that causes leaf mold disease of tomato. The tomato *Cf-9* gene confers resistance to *C. fulvum* races expressing the corresponding *Avr9* gene. The Avr9 protein is secreted by the fungus and is processed to a cystine knot peptide of 28 amino acids, which can be retrieved in intercellular washing fluid (IF) from infected leaves (De Wit and Spikman, 1982; Van den Ackerveken et al., 1993). Infiltration of Cf9 tomato or transgenic Cf9 tobacco with Avr9 leads to necrosis within 24 hr. This response is faster in tobacco than it is in tomato (Hammond-Kosack et al., 1998). Cell suspension cultures derived from Cf9 tobacco plants, when challenged with Avr9, rapidly produce active oxygen species (AOS) (Piedras et al., 1998) and activate two mitogen-activated protein (MAP) kinases (Romeis et al., 1999) and a calcium-dependent protein kinase (Romeis et al., 2000).

The mode of action of the Cf-9 protein is not known. Changes in gene expression are likely to be important for activation of defense mechanisms, and transcriptional changes have been reported in several plant–pathogen interaction systems (Rushton and Somssich, 1998). The nonhost resistance responses of parsley and tobacco cells to elicitors from cultures of *Phytophthora* spp have been particularly well studied (Somssich et al., 1989; Suty et al., 1996). However, analysis of transcriptional regulation has often focused on induction of pathogenesis-related (PR) proteins and enzymes of phenylpropanoid synthesis (Linthorst, 1991; Dixon and Paiva, 1995). *Cf-9* gene–dependent induction of chitinase and 1,3-β-glucanase expression has been documented (Wubben et al., 1996), but these responses were 4 to 8 hr after elicitation. We sought to identify genes that exhibited rapid Cf-9–dependent induction by Avr9. We used Cf9 tobacco cell cultures, which provide an amenable experimental system in which to study rapid Avr9 responses. Synchronous delivery of ligand to cells can be achieved more reproducibly in cultures than in leaves, making them ideal for biochemical and pharmacological studies (Piedras et al., 1998).

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The rapid production of AOS is one of the earliest defense responses (Lamb and Dixon, 1997). AOS may contribute directly to plant defense and also play a role in signaling, leading to the induction of defense genes (Jabs et al., 1997; Yang et al., 1997). Cf9 tobacco cell cultures provide a system in which we can distinguish between AOS-dependent and -independent events by preincubation with diphenyleneiodonium (DPI) to inhibit AOS production (Piedras et al., 1998). This study provides comprehensive expression profiling of rapid *R* gene–dependent, AOS-independent gene induction during the plant defense response. The RNA fingerprinting technique of cDNA amplified fragment length polymorphism (cDNA-AFLP) (Bachem et al., 1996, 1998) was used. Of ~30,000 cDNA fragments inspected, 290 fragments showed altered abundance within 15 to 30 min after adding Avr9. Of these, 263 were induced by Avr9 even in the presence of DPI. Some showed homology to known genes, of which many may have roles in further signaling. We also observed the induction of *Avr9/Cf-9* rapidly elicited (*ACRE*) genes in Cf9 tobacco leaves in response to Avr9 infiltration. We discuss the importance of this large set of induced genes in the defense response and in other stress responses.

RESULTS

Rapid Alterations in Cf9 Tobacco Transcript Levels in Response to Avr9

Cf9 tobacco cell cultures were treated with IF containing Avr9 (IF[Avr9$^+$]) or lacking Avr9 (IF[Avr9$^-$]) or pretreated with DPI for 5 min before adding IF(Avr9$^+$). Cells were harvested after 30 min, and RNA was extracted and used for cDNA-AFLP. In experiments conducted simultaneously, Avr9-dependent AOS production was observed. This production could be inhibited by adding DPI, as shown by Piedras et al. (1998) (data not shown). Gene expression was surveyed in two independent sets of cells using 512 primer combinations for selective amplification. A section of a typical AFLP gel used in this analysis is shown in Figure 1A. The majority of bands showed no change in intensity between the three treatments. Changes in the intensity of individual bands did not affect others in the same lane, indicating that product accumulation was not affected by the concentration of individual substrates in the reaction. The AFLP fragments ranged in length from 50 to 400 bp; for each primer combination, 50 to 70 bands were observed, with ~30,000 fragments being analyzed. Altered expression patterns were observed for 290 fragments (Figure 1B); of these, 273 were induced by Avr9. Only 4% of the changes in expression were inhibited by DPI, indicating that most rapid changes are independent of AOS.

Control experiments on 30 fragments confirmed that their differential expression required both Avr9 and *Cf-9* (Figure 1C). No induction was observed in nontransformed tobacco cell cultures. Treatment with the chemically synthesized Avr9 peptide was sufficient to produce the differential expression observed after IF(Avr9$^+$) treatment, confirming that Avr9 was responsible for the changes (Figure 1C). These controls demonstrate that the altered gene expression is *Cf-9* and Avr9 dependent.

Compilation of Sequences from Induced and Repressed cDNA Fragments

The differentially expressed fragments were excised from the gels, reamplified by polymerase chain reaction (PCR), and sequenced. DNA sequences were obtained for 260 fragments. The other sequences were a mixture of PCR products and could not be directly sequenced. The sequences were compared with those in the GenBank database using the BLAST program (Altschul et al., 1997). Sequence similarity was found for 37 induced and five repressed sequences (Table 1). These sequences were similar to those of protein kinases, transcription factors, calcium binding proteins, a protein phosphatase, a 13-lipoxygenase, and other proteins. Their roles in signal transduction suggest that many Avr9-induced proteins have signaling functions. The sequences of the majority of the fragments showed no homology to known sequences. They may represent previously uncharacterized genes, or the AFLP fragments may have been too short to reveal significant homology. The sequence data for the fragments listed in Table 1 have been submitted to GenBank, and their accession numbers are listed in Table 1.

Time-Course and Inhibitor Studies of Selected cDNA Fragments

We focused our attention on 18 cDNA-AFLP fragments induced independently of AOS. These were selected based on their homology to known genes or because they were strongly induced. Their expression patterns were examined in Cf9 cells during a 4-hr time period after IF was added. All of the cDNA fragments were induced by IF(Avr9$^+$) (Figure 2) but not by IF(Avr9$^-$) (data not shown), but their patterns of expression varied in strength and timing. Fragments 1 and 111 were rapidly but transiently induced within 15 min, with maximum expression after 30 to 60 min; expression decreased after 2 to 4 hr. Fragment 4 showed very transient expression; it was induced 30 min after elicitation, but expression had decreased by 1 hr. The other 15 fragments were also induced rapidly, but their induction was more sustained and transcript levels remained high for at least 4 hr. These patterns were reproducible in at least two independent experiments.

Most of the genes were induced in the presence of DPI, suggesting that AOS are not intermediates in the pathway to

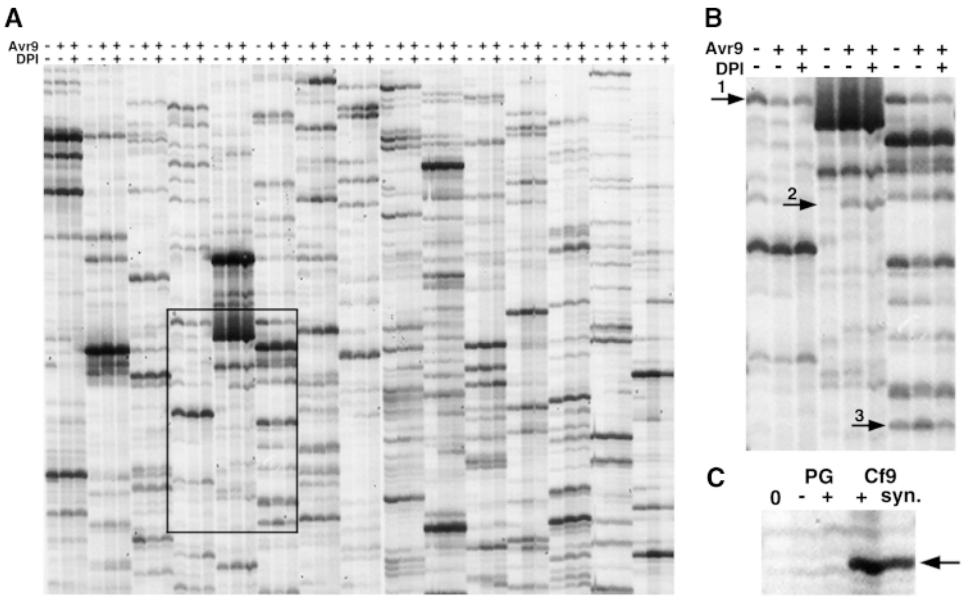

Figure 1. cDNA-AFLP Display of Avr9/Cf-9–Dependent Changes in Gene Expression.

(A) Cf9 tobacco cells were treated with IF(Avr9$^+$) or IF(Avr9$^-$) or pretreated for 5 min with DPI before addition of IF(Avr9$^+$). Cells were harvested after 30 min. Lanes are in groups of three: each group was amplified with a different pair of selective AFLP primers. (+) indicates Avr9 or DPI added; (−) indicates Avr9 or DPI not added.

(B) Enlarged view of the boxed area in **(A)**. The arrows indicate repressed (1) and induced fragments (2) and a fragment whose induction is inhibited by DPI (3). (+) indicates Avr9 or DPI added; (−) indicates Avr9 or DPI not added.

(C) Induction requires both Cf-9 and Avr9. Cell cultures of Cf9 and nontransformed tobacco (PG) were analyzed 30 min after elicitation with IF(Avr9$^+$) (+), IF(Avr9$^-$) (−), or chemically synthesized Avr9 (syn.) and compared with untreated Cf9 cells (0).

gene induction and that an alternative pathway must be involved. The protein synthesis inhibitor cycloheximide (CHX) was used to test whether induction of these 18 genes required de novo protein synthesis. Cf9 cells were pretreated for 1 hr with 5 μg/mL CHX. IF was then added, and cells were harvested after another 30 min. Treatment with CHX did not inhibit Avr9-dependent gene induction; rather, it induced the expression of these genes in the absence of Avr9 (Figure 3). This suggests that new protein synthesis is not required to induce the 18 genes tested, reinforcing the idea that their induction is an early target of Cf-9. Induction by CHX alone is typical of many early-response genes and suggests the involvement of a short-lived repressor (Koshiba et al., 1995).

Isolation of cDNA Clones Corresponding to 13 cDNA-AFLP Fragments

Sixteen out of the 18 AFLP fragments described above were used to screen a cDNA library for full-length clones (Table 2). The cDNA library was made from Cf9 tobacco cells harvested 30 min after the addition of IF(Avr9$^+$). Before using the fragments for library screening, their identity was verified by repeating the AFLP technique using three selective nucleotides (rather than two) on the MseI primer and sequencing the band excised from the gel. Repeating the AFLP is important because no part of the gel is free from other PCR products, and it is likely that more than one fragment will be excised from the gel. Increasing the number of selective nucleotides decreased the number of fragments being amplified, thereby reducing the likelihood of amplifying contaminating fragments. The sequences of these fragments matched those from the original AFLP screen for all fragments used for library screening (data not shown). The reamplified fragments were used as probes to screen the library.

The results of the library screening are summarized in Table 2. We isolated and sequenced single clones for 13 AFLP fragments. *Nicotiana tabacum* is an allotetraploid originating from two distinct diploid species (*N. sylvestris* and *N. tomentosiformis*). The clones hybridizing with AFLP fragments 31, 75, 111, 137, and 180 and the AFLP fragments themselves had mismatched nucleotides, suggesting that these clones derived from a homolog of the cDNA from which the AFLP fragment originated. Clones with a 100% match at the

Table 1. Homologies of Sequences of AFLP Fragments to Sequences in the Databases

AFLP Fragment	GenBank Accession Number	Length (bp)	Induced/ Repressed[a]	Inhibited by DPI?	Homology[b]	BLAST Score[c]
1	AF211541	151	+	No	EREBP-4 homolog from Arabidopsis (Z97343)	8e-15
4	AF211544	141	+	No	NL27 from potato (AJ009720)	3e-9
10	AF211550	144	+	No	Putative protein predicted from genome sequence of Arabidopsis (AL022140)	0.007
11	AF211551	107	+	No	S domain receptor-like protein kinase from maize (AJ010166)	0.001
31	AF211566	154	+	No	Calcium binding protein CAST from potato (L02830)	6e-19
36	AF211570	77	+	No	Putative chloroplast nucleoid DNA binding protein from Arabidopsis (AC006580)	2e-4
41	AF211574	152	−	No	Nonclathrin coat protein gamma-like protein from Arabidopsis (AL023094)	2e-19
44	AF211577	91	+	No	13-Lipoxygenase from potato (X96406)	2e-8
47	AF211580	121	+	No	ACC synthase from tobacco (AJ005002)	9e-12
52	AF211584	154	+	No	Anthocyanin-3-glucoside rhamnosyltransferase from Arabidopsis (AC006592)	6e-7
57	AF211589	208	+	No	Calmodulin-like protein from Arabidopsis (AL049862)	6e-14
64	AF211594	86	+	No	Peroxidase 3 from *Scutellaria baicalensis* (AB024439)	9e-6
76	AF211605	133	+	No	Unknown protein predicted from genome sequence of Arabidopsis (AC005623)	1e-8
84	AF211611	154	+	No	Hypothetical protein predicted from genome sequence of Arabidopsis (AL050352)	7e-4
85	AF211612	168	+	No	Hypothetical protein predicted from genome sequence of Arabidopsis (AC005397)	3e-4
97	AF211622	129	−	Yes	MAP kinase–like protein from *Selaginella lepidophylla* (U96716)	2e-6
101	AF211626	71	+	No	Similar to synaptobrevin-related protein from Arabidopsis (Z97339)	8e-4
102	AF211627	125	+	No	Putative mitochondrial protein predicted from genome sequence of Arabidopsis (AC006418)	2e-12
105	AF211630	68	−	Yes	Chloroplast genome DNA from tobacco (Z00044)	2e-19[c]
110	AF211634	79	−	Yes	Chloroplast genome DNA from tobacco (Z00044)	3e-37[c]
111	AF211635	117	+	No	DREB1A from Arabidopsis (AB007787)	1e-14
126	AF211645	141	+	No	Protein similar to WRKY transcription factors from Arabidopsis (AC011809)	3e-12
139	AF211656	147	+	No	Hypothetical protein predicted from genome sequence of Arabidopsis (AC005662)	2e-11
143	AF211660	144	+	No	Phosphate-induced phi-1 protein from tobacco (AB018441)	5e-24
146	AF211662	108	+	No	Putative protein predicted from genome sequence of Arabidopsis (AL078470)	8e-5
150	AF211666	176	+	No	Serine/threonine protein kinase isolog from Arabidopsis (U95973)	6e-10
157	AF211672	146	+	No	Similarity to transcription factor TINY from Arabidopsis (AC003979)	5e-6
164	AF211679	103	+	No	Expressed sequence tag from Arabidopsis (AC006577)	0.002
175	AF211690	118	+	No	Hexose transporter protein from tomato (AJ010942)	5e-7
215	AF211724	89	+	No	Flavanol 3-sulfotransferase from *Flaveria bidentis* (U10275)	0.002
216	AF211725	96	+	No	WPK4 protein kinase, induced by cytokinins, light and sucrose, from wheat (AB011670)	5e-8
223	AF211732	105	+	No	Polygalacturonase 1 β chain from tomato (M98466)	3e-10
228	AF211736	174	+	No	Auxin-induced protein 15A from soybean (S44172)	1e-13
231	AF211738	150	+	No	Hypothetical protein predicted from genome sequence of Arabidopsis (AC002396)	3e-7
236	AF211741	104	+	No	Serine/threonine protein kinase from Arabidopsis (AL022603)	2e-4
246	AF211749	118	+	No	Putative host response protein from Arabidopsis (AL049481)	5e-6
256	AF211758	147	+	No	Receptor-like protein kinase from Arabidopsis (M84658)	5e-7
258	AF211760	154	−	No	Proton-ATPase β subunit from tobacco (X61319)	3e-22
264	AF211766	131	+	No	Putative protein kinase from Arabidopsis (AC005970)	2e-12
267	AF211767	106	+	No	Putative protein predicted from genome sequence of Arabidopsis (AL021749)	6e-4
284	AF211780	136	+	No	Protein phosphatase 2C from alfalfa (Y11607)	2e-15
288	AF211784	158	+	No	Expressed sequence tags similar to glycosyl transferases from Arabidopsis (AC002062)	2e-14

[a] Induction (+) or repression (−) by Avr9.

[b] GenBank accession numbers of sequences homologous to AFLP fragment are in parentheses.

[c] All are BLASTX scores except for 105 and 110, which are BLASTN scores.

nucleotide level with the AFLP fragment were obtained for the remaining eight fragments. When AFLP fragment 111 was used as a probe, two hybridizing clones were isolated, ACRE-111A and ACRE-111B, with a 99 and 94% match at the nucleotide level with the AFLP fragment, respectively. In some cases, cDNAs of different lengths with different polyadenylation sites were obtained. The genes were named *ACRE* genes, followed by the number corresponding to the AFLP fragment used for library screening.

Seven *ACRE* transcripts encoded proteins with similarity to sequences in the GenBank databases (percentage identity; amino acids in Table 2). The remainder showed no homology and represent previously unidentified gene products. ACRE-1, ACRE-111A, and ACRE-111B have amino acid sequences homologous to those of the ethylene response element binding protein (EREBP) transcription factors. ACRE-1 was similar to Arabidopsis ERF6 and tobacco EREBP4, which is induced by ethylene and wounding (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1998). The amino acid identity over the entire protein was low (49% for AtERF6) but much higher (78% for AtERF6) in the DNA binding domain, which is conserved throughout the EREBP family. ACRE-111A and ACRE-111B were most similar to Arabidopsis DREB1A (for dehydration-responsive element binding protein), which is induced by cold stress (Liu et al., 1998). Both ACRE-1 and ACRE-111A had small open reading frames (uORFs) upstream of the major ORF. ACRE-1 had three uORFs of 78, 9, and 18 bp, and ACRE-111A had two uORFs of 15 and 18 bp. uORFs have been described in other transcripts, and although present in <10% of eukaryotic mRNAs, they are more common in regulatory genes and have been shown to repress translation of the main ORF (Wang and Wessler, 1998).

ACRE-4 is 82% identical to the N resistance protein from tobacco. This homology extends throughout the N terminus of the protein but stops before the leucine-rich repeats. The predicted structure of ACRE-4 is similar to the truncated N polypeptide, Ntr, a product of alternative splicing (Whitham et al., 1994). No clone was identified encoding a full-length N-like protein.

ACRE-31 is 83% identical to CAST, a calcium binding protein of unknown function from potato (Gellatly and Lefebvre, 1993). It has three putative EF hand calcium binding domains.

ACRE-132 is homologous to a number of zinc finger proteins of the RING-H2 (for really interesting new gene) class E and is most homologous to the auxin-induced *ATL2* gene product of Arabidopsis (Martínez-García et al., 1996). It has the conserved RING-H2 motif and a hydrophobic domain at the N terminus (predicted to be a membrane anchor), which are characteristic of this class of RING-H2 proteins (Saurin et al., 1996; Jensen et al., 1998).

ACRE-75, ACRE-137, ACRE-146, ACRE-169, and ACRE-231 are similar to hypothetical proteins from Arabidopsis identified during genome sequencing. The ACRE-231 sequence also has 22% amino acid identity to glycosyl transferases of bacteria involved in lipopolysaccharide biosynthesis. *ACRE-65*, *ACRE-75*, *ACRE-169*, *ACRE-180,* and *ACRE-194* encode small peptides of unknown function of 97, 139, 124, 102, and 156 amino acids, respectively. The sequences of the ACRE cDNA clones have been submitted to GenBank as accession numbers AF211527 to AF211540.

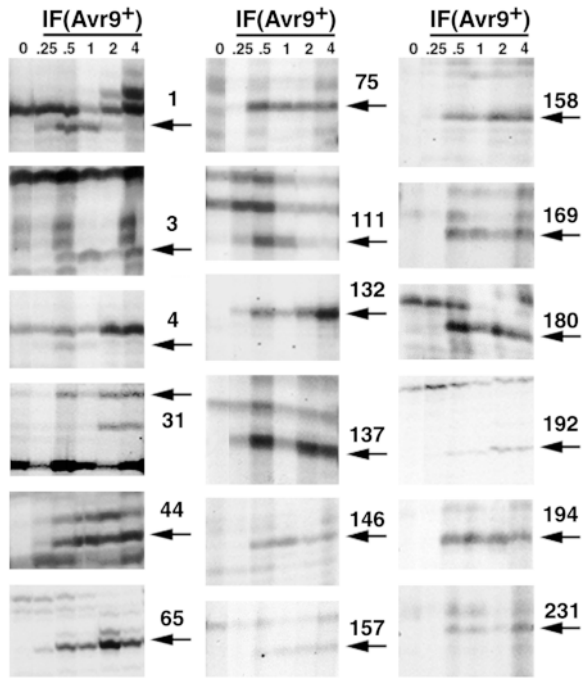

Figure 2. Expression Patterns of 18 Induced Genes.

Cf9 tobacco cells were treated with IF(Avr9$^+$) for the times indicated above the gels (in hours) before harvesting. Expression was examined by using cDNA-AFLP analysis. Each gel is labeled with the number of the AFLP fragment, and the corresponding band is labeled with an arrow.

RNA Gel Blot Analysis Verifies Gene Induction in Cell Cultures

To verify the expression patterns observed after cDNA-AFLP analysis, RNA gel blot analysis was performed using probes derived from four ACRE cDNAs. For ACRE-31, ACRE-146, and ACRE-169, the full-length cDNAs were used as probes. To avoid cross-hybridization with related EREBPs, we used a 600-bp fragment from the 5′ end of the ACRE-1 cDNA. This fragment was used because it did not include the region encoding the conserved DNA binding domain. The induction pattern we observed in cell cultures by using this method was the same as that seen when cDNA-AFLP analysis was conducted (Figures 4A and 4B).

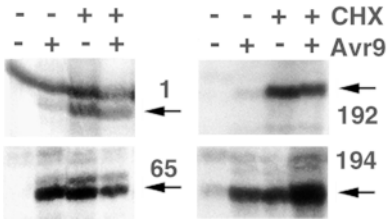

Figure 3. CHX Treatment Does Not Block Avr9-Dependent Gene Induction, but It Induces Expression in the Absence of Avr9.

Cf9 tobacco cells were pretreated with 5 μg/mL CHX or ethanol for 1 hr, and then IF(Avr9$^+$) or IF(Avr9$^-$) was added; cells were harvested after another 30 min. Expression was examined by using cDNA-AFLP analysis. (+) indicates Avr9 or CHX added; (−) indicates Avr9 or CHX not added. Each gel is labeled with the number of the AFLP fragment, and the corresponding band is labeled with an arrow.

ACRE Gene Expression in Leaves: Transient Nonspecific Induction and Sustained Avr9-Dependent Induction

We investigated the expression patterns of *ACRE-1*, *ACRE-31*, *ACRE-146*, and *ACRE-169* in leaves of nontransformed (data not shown) or Cf9 tobacco after injection with IF. Surprisingly, the *ACRE* genes were rapidly but transiently induced in leaves after 30 min, regardless of the genotype or elicitor used. The induction by Avr9 was stronger and/or more sustained (Figure 4C). Additional experiments were conducted in which the leaves were cut with a razor blade and either injected with water, $MgCl_2$, or $MgSO_4$ or not injected and harvested after 30 min. Induction of 18 AFLP fragments was investigated by using cDNA-AFLP analysis (Figure 5), and induction of *ACRE-1*, *ACRE-31*, *ACRE-146*, and *ACRE-169* was also examined by RNA gel blot analysis (Figure 4D). Infiltration of leaves with any solution produced strong but transient (peaking after 30 min) induction of all AFLP fragments inspected. Cutting the leaves without infiltration induced all fragments, except for fragments 3, 65, 157, 169, and 192. Induction by cutting was in most cases weaker than that elicited by infiltration but provoked a similar response for fragments 1, 31, 44, 75, 111, and 194. These results are consistent with other data suggesting an overlap between the responses induced by different stresses, such as fungal elicitor, wounding, and flooding of the apoplast in plants (Romeis et al., 1999). Recent data from mammals also suggest that overlapping sets of genes are induced by the different signal pathways activated by growth factor receptors (Fambrough et al., 1999).

Hin1, Hsr203J, and EREBP1 Transcripts Also Accumulate in Response to Avr9

Using RNA gel blot analysis, we were able to determine whether other defense-induced genes were rapidly elicited in the *Cf-9*/Avr9 system. *Hin1* (for harpin-induced) and *Hsr*203J (for hypersensitivity-related) are reported to be rapidly induced by bacterial harpins and *Avr* gene products during the hypersensitive response of tobacco (Pontier et

Table 2. Summary of Clones Obtained from the Cf9 Tobacco cDNA Library

ACRE Number[a]	GenBank Accession Number	cDNA Length (bp)[b]	Percentage Match to AFLP Fragment Used for Screening	Homology of Translated Sequences of cDNA Clones to Amino Acid Sequences in the Databases[c]	Percentage Identity (Amino Acids)	Peptide Length (Amino Acids)
1	AF211527	1148/1166	100	AtERF6 from Arabidopsis (AB013301)	49	237
4	AF211528	2833	100	N resistance protein from *N. glutinosa* (U15605)	82	536
31	AF211529	967/972	99	CAST from potato (L02830)	83	205
65	AF211539	475	100	None		97
75	AF211540	633/733	94	Hypothetical protein F12K8.18 from Arabidopsis (AC006551)	35	139
111A	AF211530	992	99	DREB1A from Arabidopsis (AB007787)	66	184
111B	AF211531	956/1000	94	DREB1A from Arabidopsis (AB007787)	65	219
132	AF211532	956	100	RING-H2 protein ATL3 from Arabidopsis (AF132013)	41	256
137	AF211537	1171	97	Unknown protein F23M19.3 from Arabidopsis (AC007060)	27	304
146	AF211533	789/927	100	Unknown protein F5F19.20 from Arabidopsis (AC001645)	50	182
169	AF211534	867	100	Unknown protein F20D23.16 from Arabidopsis (AC007651)	48	124
180	AF211538	621	98	None		102
194	AF211535	651	100	None		156
231	AF211536	1382/1396	100	Unknown protein F20P5.18 from Arabidopsis (AC002062)	63	353
				Glycosyl transferase *Neisseria gonorrheae* (U14554)	22	

[a] ACRE number corresponds to the number of the AFLP fragment used for library screening.
[b] Where two cDNA lengths are listed, they come from different clones of the same cDNA that had different polyadenylation sites.
[c] GenBank accession numbers are in parentheses.

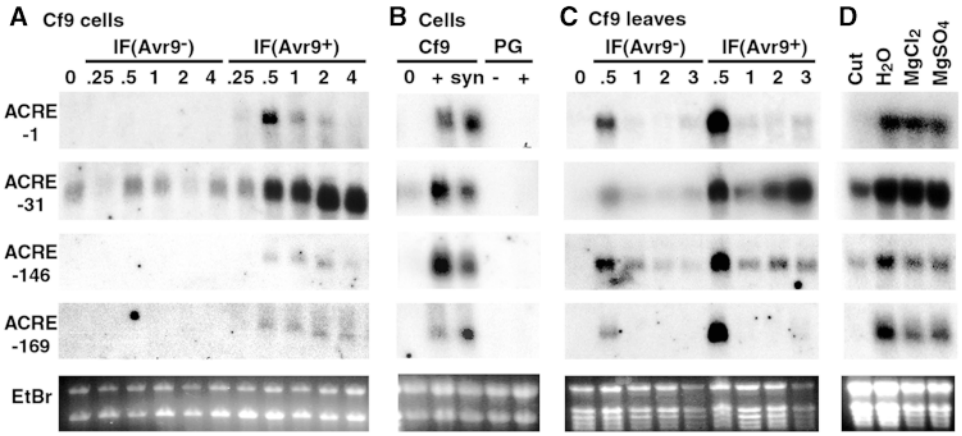

Figure 4. RNA Gel Blot Analysis Confirms *ACRE* Gene Induction in Cf9 Cell Cultures.

ACRE genes are also induced in leaves by Avr9 and infiltration of fluids. Total RNA (5 to 10 μg) extracted from cell cultures or leaves was used for RNA gel blot analysis. Loading of gels is shown by ethidium bromide (EtBr) staining. The same blots were probed with four *ACRE* cDNAs.
(A) and **(C)** Cf9 cell cultures **(A)** or leaves **(C)** were treated with IF(Avr9$^+$) or IF(Avr9$^-$) for the times indicated (given above blots in hours).
(B) Cf9 or nontransformed (PG) cell cultures were analyzed 30 min after treatment with IF(Avr9$^+$) (+), IF(Avr9$^-$) (−), or chemically synthesized Avr9 (syn) and compared with untreated Cf9 cells (0).
(D) Tobacco leaves were cut with a razor blade and either injected with water (H_20), $MgCl_2$, or $MgSO_4$ or not injected (Cut) and harvested after 30 min.

al., 1994; Gopalan et al., 1996). The tomato homolog of *Hsr*203J is induced by Avr9 in Cf9 plants within 3 hr (Pontier et al., 1998).

In Cf9 tobacco cells, *Hin1* and *Hsr*203J were expressed at a low constitutive level and were strongly induced by Avr9 (Figure 6A). Maximal induction of *Hsr*203J was after 2 hr. Its induction was later than that of the *ACRE* genes. The *Hin1* transcript accumulated to the same level 30 min after the addition of either IF(Avr9$^-$) or IF(Avr9$^+$); however, induction by IF(Avr9$^-$) was transient, whereas induction by IF(Avr9$^+$) continued for at least 4 hr. The late induction of *Hsr*203J and the accumulation of *Hin1* at 30 min after either treatment explain why they were not detected in our AFLP screen for rapidly induced genes.

In Cf9 tobacco leaves, there was also some constitutive expression of both genes, and the pattern of induction by IF(Avr9$^+$) was similar to that observed in cells. The *Hin1* transcript accumulated after 15 min and *Hsr*203J after 2 hr (Figure 6B). Some induction by infiltration of IF(Avr9$^-$) and water or buffer was also observed after 30 min (Figures 6B and 6C).

To test further the role of EREBPs in Cf-9–mediated defense, we examined mRNA accumulation of *EREBP1*, which is induced in response to many stresses, including ethylene, salicylic acid, and wounding (Ohme-Takagi and Shinshi, 1995; Horvath et al., 1998; Suzuki et al., 1998). It is also induced by *Pseudomonas syringae* pv *tabaci* expressing the *avrPto* avirulence gene in tobacco plants expressing the *Pto* resistance gene (Zhou et al., 1997). *EREBP1* was expressed at a low level in untreated Cf9 cell cultures (Figure 6A). The transcript levels 30 min after treatment with IF(Avr9$^-$) or IF(Avr9$^+$) were the same, explaining why *EREBP1* was not identified in the original AFLP screen, despite having suitable restriction sites. In leaves, *EREBP1* expression was induced within 30 min after leaf infiltration, which is consistent with the results of Suzuki et al. (1998) (Figures 6B and 6C). However, induction by Avr9 was stronger and more prolonged than induction by wounding (Figure 6B).

DISCUSSION

To understand the Cf-9–mediated plant defense response, we undertook a comprehensive analysis of rapid Cf-9/Avr9–dependent changes in gene expression. We identified genes induced early in the defense response because these are most likely to have regulatory and signaling functions. In other studies, analysis of early gene induction has been performed using elicitation with fungal proteins that are involved in non-host interactions. These include the addition of the peptide elicitor Pep-25 from *P. sojae* to parsley cell cultures, a well-established model system for a non-host interaction, and cryptogein from *P. cryptogea* to tobacco cells (Somssich et al., 1989; Suty et al., 1996). A number of rapidly induced genes were observed; some had maximum expression 30 to 60 min after the elicitor was added. However,

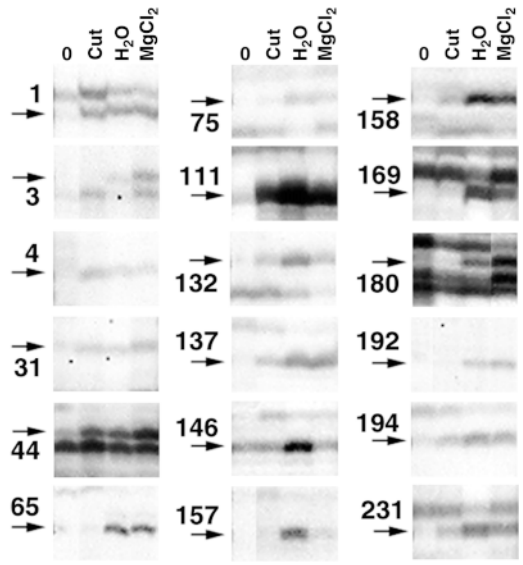

Figure 5. *ACRE* Genes Are Induced in Leaves by Avr9 and Infiltration of Fluids.

Tobacco leaves were cut with a razor blade and either injected with water (H_20) or $MgCl_2$ or not injected (Cut), harvested after 30 min and compared with untreated leaves (0). Expression was examined by using cDNA-AFLP analysis. Each gel is labeled with the number of the AFLP fragment, and the corresponding band is labeled with an arrow.

few have addressed whether the same genes are induced in a gene-for-gene system.

We used cDNA-AFLP analysis to detect immediate early gene expression during the *Cf-9*– and Avr9-dependent plant defense response in Cf9 tobacco cell cultures. The response of these cultures to Avr9 elicitation has been well characterized (Piedras et al., 1998; Romeis et al., 1999). Cell cultures provide a more reproducible system than do leaves for the analysis of induced responses. The rapid production of AOS is characteristic of plant defense responses, and it has been suggested that AOS play a role in signaling, leading to changes in gene expression (Jabs et al., 1997; Yang et al., 1997). The Avr9-elicited production of AOS is abolished by DPI (Piedras et al., 1998), which allowed us to investigate the contribution of AOS to *Cf-9*–dependent gene expression. Many genes are rapidly induced in Cf9 cell cultures by elicitation with Avr9. This induction is dependent on the presence of *Cf-9* and Avr9 and for most induced genes does not require the presence of AOS. CHX did not inhibit this induction. On the contrary, it strongly induced *ACRE* gene expression even in the absence of Avr9. This is typical of immediate early response genes (Koshiba et al., 1995) and suggests regulation by a rapidly turned over repressor protein. In comparison to *Hin1* and *Hsr*203J, which were previously described as rapidly induced genes, it became clear that *ACRE* gene induction is a very early response. This suggests that *ACRE* genes are involved in the initial development of the defense response.

Advantages of cDNA-AFLP Analysis

By using cDNA-AFLP analysis, we were able to survey transcriptional changes with no prior assumptions about which genes might be induced or repressed. cDNA-AFLP analysis gave reproducible results that were confirmed using RNA gel blot analysis (Bachem et al., 1996, 1998). Amplification of fragments from constitutively expressed genes provided internal control bands with every primer combination. cDNA-AFLP analysis has advantages over other RNA fingerprinting techniques and DNA chip-based approaches (McClelland et al., 1995; Baldwin et al., 1999). DNA chips can only be used to analyze the expression of cloned genes (Kehoe et al., 1999), whereas cDNA-AFLP analysis can reveal altered expression of any gene that carries suitable restriction sites. With the enzyme combination chosen, ~30,000 cDNA fragments were inspected, of which 290 were differentially expressed; therefore, ~1% of the transcripts rapidly alter in abundance after the addition of the Avr9 elicitor. To visualize a cDNA by using an AFLP, it must contain ApoI-MseI fragments of 50 to 400 bp. We inspected the sequence of 50 tobacco mRNAs and found that 76% contained suitable ApoI-MseI fragments. This means that 76% of genes expressed could potentially be visualized by using this enzyme combination. Also, tobacco is an allotetraploid between two polymorphic species; therefore, if one copy of a gene cannot be visualized, then there is still a chance of seeing another copy. Thus, it seems likely that a large proportion (>75%) of the transcripts were inspected for their response to the addition of Avr9.

A Limited Role for AOS in Rapid Defense Gene Induction

Most of the early *Cf-9*– and Avr9-dependent changes in gene expression were not inhibited by DPI and therefore are not dependent on AOS. Studies with other systems have concentrated mainly on genes with roles in phytoalexin production or cellular protection. In soybean, H_2O_2 induces expression of genes required for cellular protection but not those that encode phenylalanine ammonia lyase or chalcone synthase, which are involved in phytoalexin synthesis (Levine et al., 1994). In parsley cell cultures challenged with the peptide elicitor Pep-13 from *P. sojae*, treatment with DPI, but not catalase, inhibited both phytoalexin production and the induction of many defense genes, indicating a requirement for superoxide rather than H_2O_2 (Jabs et al., 1997). It is feasible that although the oxidative burst is not required for rapid induction of *ACRE* genes, it may play a role in the expression of genes that respond at later times when AOS levels are higher.

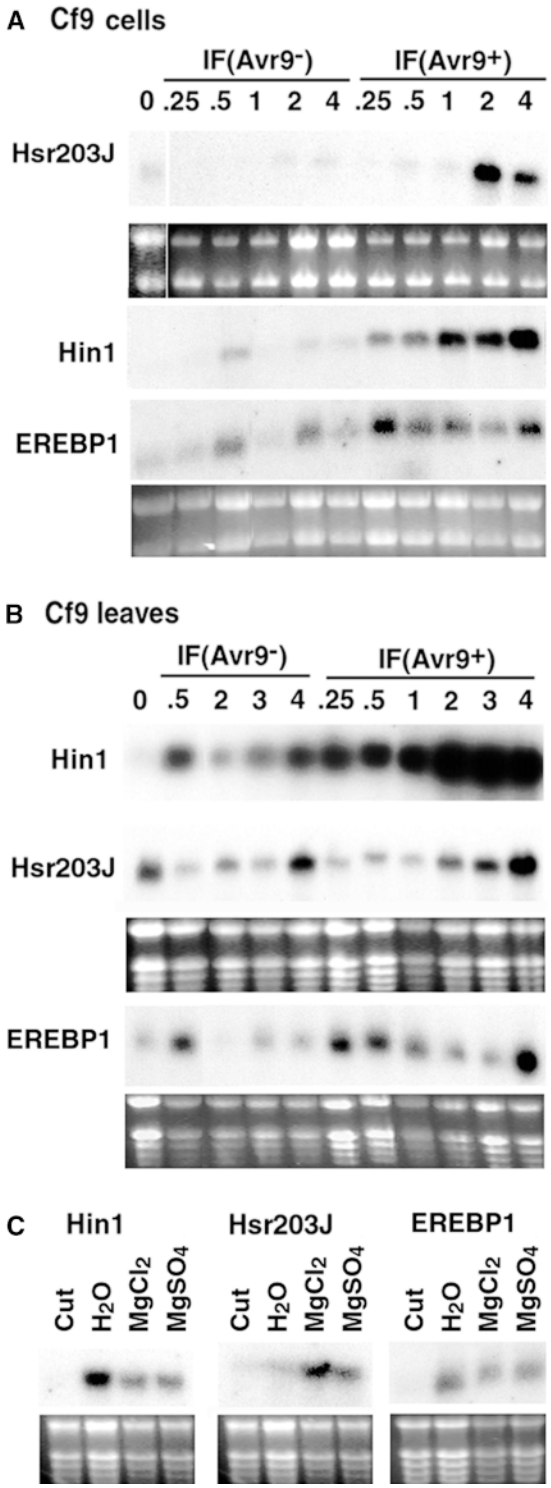

Figure 6. *Hin1*, *Hsr*203J, and *EREBP-1* Expression Is Induced by Avr9/Cf-9 and Infiltration of Fluids into Leaves.

Total RNA (5 to 10 μg) extracted from cell cultures or leaves was used for RNA gel blot analysis. Loading of gels is shown by ethidium bromide staining.

(A) and **(B)** Cf9 cell cultures **(A)** and leaves **(B)** were treated with IF(Avr9$^+$) or IF(Avr9$^-$) for the times indicated (given above blots in hours).

(C) Tobacco leaves were cut with a razor blade and either injected with water (H_2O), $MgCl_2$, or $MgSO_4$ or not injected (Cut) and harvested after 30 min.

ACRE Gene Products and Their Possible Roles in Signal Transduction

ACRE-65, ACRE-75, ACRE-169, *ACRE-180*, and *ACRE-194* encode small peptides of <160 amino acids. A number of small peptides are induced during the defense response. Some of these have antimicrobial properties, for example, defensins and thionins (Broekaert et al., 1997). However, these small ACRE proteins are unlikely to be defensins or thionins because they are not cysteine rich and have no apparent signal peptide. ACRE-137, ACRE-146, and ACRE-231 are similar to hypothetical proteins predicted from the genome sequence of Arabidopsis. These ACRE proteins may play roles in signaling or other aspects of the defense response, but their function is unknown.

Some of the induced genes are candidates for components of signaling pathways, which may be responsible for induction of other defense responses. Alternatively, they may encode proteins involved in pathways leading to gene expression that are induced by a positive feedback pathway to replace the protein as it is degraded. Protein phosphorylation cascades play an important role in many signaling pathways. The sequence homology of seven AFLP fragments to sequences of protein kinases and one to a phosphatase (Table 1) is therefore not unexpected.

AFLP fragment 44 is similar to cDNAs encoding 13-lipoxygenases, which are enzymes that catalyze an early step in the synthesis of jasmonic acid, which accumulates after wounding and herbivore attack (Creelman and Mullet, 1997). Jasmonic acid has been shown also to be important for resistance to fungal pathogens. For example, the Arabidopsis mutant *coi1* (for coronatine insensitive), which is blocked in its response to jasmonic acid, shows enhanced susceptibility to *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998). The incompatible interaction between tobacco plants and *P. parasitica* var *nicotianae* is abolished in plants expressing antisense lipoxygenase (Rancé et al., 1998).

Calcium channel blockers, such as lanthanum, interfere with Cf-9 signaling, suggesting that calcium influx is required for the oxidative burst (Piedras et al., 1998). ACRE-31, a putative calcium binding protein with three EF hands, may be involved in signaling cascades, and this suggests that calcium is also involved in other responses.

ACRE-132 is a putative zinc finger protein of the RING-H2 subfamily. The cellular functions of RING proteins are diverse, but they are often involved in formation of multiprotein complexes (Saurin et al., 1996; Jensen et al., 1998). ACRE-132 has a hydrophobic domain at the N terminus that may function as a membrane anchor, so it might be part of a multiprotein complex anchored in the plasma membrane.

The sequence of ACRE-4 is 82% identical to Ntr, the product of the differentially spliced transcript N_L from the *N* resistance gene. The induction of an *N* gene homolog during *Cf-9*–mediated resistance is particularly intriguing. Both ACRE-4 and N contain a putative nucleotide binding site and a domain with homology to Toll and the interleukin-1 receptor (Whitham et al., 1994). The ratio of the alternatively spliced *N* transcripts changes after inoculation with tobacco mosaic virus (TMV) and plays a critical role in the TMV resistance response. The N_S transcript, which encodes the full-length N protein, is more prevalent before and during the first 3 hr of infection with TMV. The N_L transcript, which encodes the truncated N protein (Ntr), is more prevalent 4 to 8 hr after infection with TMV (Dinesh-Kumar and Baker, 2000). The induction of an *N* homolog by way of a *Cf-9*–dependent pathway suggests that the two classes of resistance gene may have some downstream signaling components in common and that there could be interaction between components of pathways activated by different classes of resistance protein.

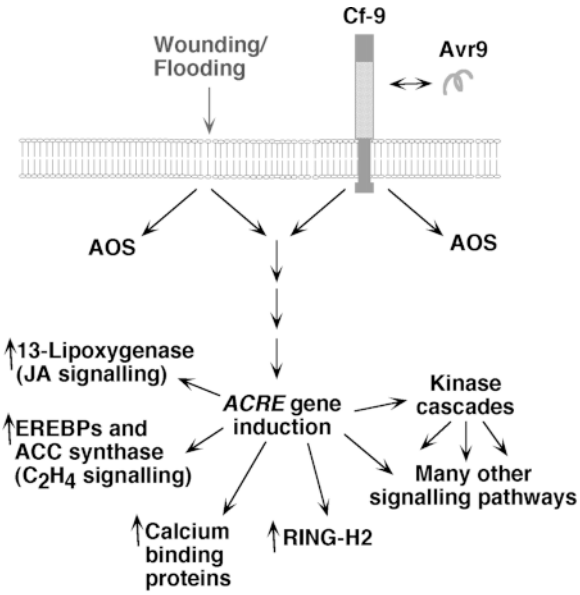

Figure 7. Model for the Role of *ACRE* Genes in Plant Stress Responses.

Upward pointing arrows indicate elevated protein levels. The double-headed arrow indicates a possible interaction between the Cf-9 and Avr9 proteins.

Importance of EREBP Transcription Factors in Defense

The amino acid sequences of ACRE-1 and ACRE-111A are 41 and 66% identical to those of EREBP4 and DREB1A, respectively. These are members of a large family of transcription factors thought to control expression of many genes during the defense responses induced by a number of biotic and abiotic stresses (Riechmann and Meyerowitz, 1998). Tobacco EREBP1 to EREBP4 bind GCC boxes in PR gene promoters, and their transcript levels are induced by C_2H_4 and wounding (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1998). DREB1A, DREB1B, and DREB1C bind the CRT/DRE sequence found in promoters of genes regulated by cold and drought (Liu et al., 1998). The rapid but transient induction of *ACRE-1* and *ACRE-111A* by Avr9 suggests that they are required for induction of other defense response genes. ACRE-1 and ACRE-111A are likely to bind different promoter elements, the GCC box and CRT/DRE box respectively, based on their homology to known proteins. This suggests that they may regulate different sets of response genes required for plant defense. It has been suggested that EREBPs regulate the expression of the genes encoding them via interaction with GCC boxes in their promoters (Solano et al., 1998). Three tomato EREBPs, Pti4, Pti5, and Pti6, were identified as proteins that interact with the Pto resistance protein (Zhou et al., 1997). Pto is a protein kinase, which suggests that EREBP activity could be controlled by phosphorylation as well as by changes in the level of transcription. C_2H_4 plays an important role in pathogen resistance. For example, tobacco transformed with *etr1-1* (for ethylene resistant) from Arabidopsis, conferring dominant C_2H_4 insensitivity, shows reduced basic *PR* gene expression and loses non-host resistance to soil-borne fungi (Knoester et al., 1998). C_2H_4 alone may not be sufficient for resistance; for *A. brassicicola* resistance in Arabidopsis, the C_2H_4 and jasmonic acid pathways must both be triggered (Penninckx et al., 1998). The translated sequence of another induced AFLP fragment is homologous to 1-aminocyclopropane-1-carboxylase (ACC) synthase, a key enzyme in C_2H_4 synthesis. C_2H_4 accumulates in Cf9 tomato 9 to 12 hr after injection with IF(Avr9$^+$) (Hammond-Kosack et al., 1996). Increases in C_2H_4 also induce the expression of *EREBP* genes; therefore, this may amplify or prolong the *Cf-9*–mediated defense response by inducing the expression of the same or additional *EREBP* genes.

Other examples of rapid induction of transcription factors during the defense response have been reported. Treatment of parsley cells with the fungal elicitor Pep25 induces a rapid but transient increase in *WRKY1* (named after the conserved amino acid sequence WRKY) and *WRKY3* expression (Rushton et al., 1996). These bind elicitor-responsive W boxes in the parsley *PR-1* promoter, suggesting that they play a key role in signal transduction. One AFLP fragment

with homology to *WRKY* genes was identified during our survey of Avr9-induced genes, suggesting that WRKYs also play a role in Cf-9–mediated defense responses. Basic leucine zipper (bZIP) transcription factors are also involved in the defense response. Recently, several bZIP proteins were identified that interact with NPR1 (for nonexpressor of PR genes) and bind the *PR-1* promoter, providing a direct link from a key signaling intermediate downstream of many *R* genes to a transcription factor (Zhang et al., 1999). We did not identify any bZIP transcription factors. However, the soybean bZIP, G/HBF-1, which binds the chalcone synthase promoter, is activated by phosphorylation and not by changes in expression (Dröge-Laser et al., 1997).

Functional Significance of Expression Profiling Data

Knowledge of the expression patterns of genes can provide important information about their function. The large sets of genome-wide expression data from DNA microarrays are often studied by using cluster analysis (Bassett et al., 1999). Cluster analysis uses statistical algorithms to group sets of genes with similar patterns of expression across a number of treatments or tissue types. In a clustering analysis of 2467 yeast genes of known function, many genes that share similar functions clustered together over several treatments (Eisen et al., 1998). When the analysis was performed using all of the ~6200 yeast genes, the genes of known function clustered in the same pattern, but these clusters were expanded by the addition of uncharacterized genes. It has been inferred from such studies that the genes of unknown function that react in the same way as genes of known function have similar roles in the response to a particular stimulus. Furthermore, cluster analysis allows the grouping of different stimuli that elicit similar patterns of gene expression. This provides insight into the biological mechanisms of the responses to different stimuli but also raises new questions about how an organism decodes this to give a unique response.

In this study, we have performed a "poor man's cluster analysis." A large set of genes was identified whose members respond to a gene-for-gene stimulus in cell cultures. These could be grouped according to whether they were induced or repressed by Avr9 and whether this change in expression required AOS. The induction of *ACRE* genes by Avr9 was verified in leaves; during these experiments, it was observed that *ACRE* gene expression was also transiently induced by mechanical stress. The induction of 18 AFLP fragments by mechanical stress was therefore examined by cDNA-AFLP analysis. Of these 18 genes that were induced with gene-for-gene specificity in cell cultures, all were induced by mechanical stress in leaves. This indicates that even these very early *R* gene–dependent transcriptional responses are not specific to *R* gene function but are shared by responses to other stresses. This observation is important for the interpretation of gene induction data from other plant pathogen systems and in response to abiotic stress.

Significance of Common Responses to Different Stimuli

The *ACRE* genes were induced by cutting leaves with a razor blade and infiltrating liquid into the apoplast and by using Avr9 (Figure 5), suggesting a surprising degree of overlap between the responses elicited by *Cf-9*/Avr9 and other stresses. Other evidence for overlap in responses comes from studies on the activation of MAP kinases. Two MAP kinases, wound-induced protein kinase and salicylic acid–induced protein kinase, are rapidly induced in Cf9 tobacco by Avr9 (Romeis et al., 1999). These are also induced by a number of other stresses, including wounding, salicylate, and TMV infection (Scheel, 1998; Zhang and Klessig, 1998; Romeis et al., 1999). In mammalian cells, the numerous signal pathways activated by growth factor receptors induce many of the same genes, and the transcriptional responses to different growth factors overlap (Fambrough et al., 1999; Pawson and Saxton, 1999). The final responses to Avr9 and to mechanical stress are different. Both MAP kinase and *ACRE* gene induction are more transient after mechanical stress than after elicitation with Avr9, suggesting that the differences in final outcome may be achieved by differences in the duration and magnitude of the activation of the same pathway components and gene inductions.

A model for the role of *ACRE* genes during the defense response is proposed in Figure 7. Recognition of Avr9 in Cf9 plants results in the rapid production of AOS and induction of *ACRE* genes. These appear to be mediated by two different pathways because induction of most *ACRE* genes does not require AOS. The pathway leading to *ACRE* gene induction is also activated by wounding. ACRE proteins are candidates for components of signaling pathways involved in the activation of later defense responses.

This comprehensive survey of *Cf-9*–dependent gene induction has identified a number of genes that are induced during the plant defense response independently of AOS. There are two routes for further work. First, *ACRE* genes need to be studied to determine their function in the plant defense response by using reverse genetics. Second, the transcriptional regulation of the *ACRE* genes needs to be investigated. What transcription factors regulate *ACRE* gene expression, and how is *Cf-9*–dependent activation of these transcription factors achieved? The answers to these questions will provide further insight into inducible cellular defense mechanisms.

METHODS

Plant and Cell Culture Conditions and Treatments

Plants (*Nicotiana tabacum* cv Petite Havana and transgenic *Cf-9*–expressing tobacco) were grown as described by Romeis et al. (1999). Intercellular washing fluid (IF) was obtained from cultivar Petite Havana plants expressing *Avr9* (IF[Avr9$^+$]) or nontransformed

(IF[Avr9$^-$]) (De Wit and Spikman, 1982; Hammond-Kosack et al., 1994). Leaves were infiltrated with IF from a syringe barrel through small razor blade cuts. At given times, two leaf panels were cut from the injected area and frozen in liquid nitrogen.

Tobacco cell cultures were maintained as described by Piedras et al. (1998). Before use, cells were washed, and aliquots of 2 g of cells in a 40-mL assay buffer (5 mM MES, pH 6.0, 175 mM mannitol, 0.5 mM $CaCl_2$, and 0.5 mM K_2SO_4) were dispensed into single flasks and left to equilibrate for 3 hr at 22°C. To each flask, 150 μL of IF(Avr9$^+$) or IF(Avr9$^-$) was added, and at times given in the figures, two 2-g aliquots of cells were harvested by filtration and frozen in liquid nitrogen. The synthetic Avr9 peptide was used at a final concentration of 10 nM. To study the effects of inhibitors, we pretreated cells before elicitation for 5 min with 1 μM diphenyleneiodonium (DPI) or for 1 hr with 5 μg/mL (17.7 μM) cycloheximide (CHX). Compounds were from Sigma.

Measurement of Reactive Oxygen Species

Aliquots (0.2 mL) of all cell samples were tested for active oxygen species production using the ferricyanide-catalyzed oxidation of luminol (Schwacke and Hager, 1992).

RNA Extraction and cDNA Synthesis

Total RNA was extracted according to Logemann et al. (1987). Poly(A) RNA was isolated from 150 μg of total RNA using oligo(dT) coupled to paramagnetic beads (Dynal A.S., Oslo, Norway). cDNA was synthesized using Expand reverse transcriptase (Boehringer Mannheim), RNase H, and DNA polymerase I.

Template Preparation, Amplified Fragment Length Polymorphism Reactions, and PAGE Analysis of Products

The template for cDNA-AFLP was prepared according to Vos et al. (1995) and Bachem et al. (1996), using ApoI and MseI. Sequences of primers and adapters used for amplified fragment length polymorphism (AFLP) reactions are as follows (N can be any nucleotide): ApoI adapter top strand, 5′-CTCGTAGACTGCGTACC-3′; ApoI adapter bottom strand, 5′-AATTGGTACGCAGTCTAC-3′; MseI adapter top strand, 5′-GACGATGAGTCCTGAG-3′; MseI adapter bottom strand, 5′-TACTCAGGACTCAT-3′; ApoI preamplification primer, 5′-CTCGTAGACTGCGTACCAATT-3′; ApoI selective amplification primers, 5′-GACTGCGTACCAATT(C/T)NN-3′; MseI preamplification primer, 5′-GACGATGAGTCCTGAGTAA-3′; and MseI selective amplification primers, 5′-GATGAGTCCTGAGTAANN-3′. AFLP reactions were performed according to Bachem et al. (1996), except that a mixture of Taq and Pfu DNA polymerases (160:1 units) was used for polymerase chain reaction (PCR) in a final volume of 10 μL. Selective amplification products were separated on a 5% polyacrylamide gel run at 100 W until the bromophenol blue reached the bottom. Gels were dried onto 3MM Whatman paper (Whatman, Maidstone, UK) and positionally marked before exposing to Kodak Biomax film (Sigma) for 2 days.

Isolation and Sequencing of Fragments

Films were aligned with markings on the gels. The bands of interest were marked, cut out with a razor blade, and incubated in 150 μL of TE (10 mM Tris, pH 7.5, and 1 mM EDTA, pH 8.0) overnight at 37°C. AFLP fragments were recovered by PCR under the same conditions as used for the preamplification. The dideoxy termination method (Sanger et al., 1977) was used for sequencing, and sequences were determined using an automated sequencer. Nucleotide sequences and translated sequences were compared with nucleotide and protein sequences of the GenBank nonredundant databases and sequences of the expressed sequence tag databases by using the BLAST sequence alignment program (Altschul et al., 1997).

Construction and Screening of cDNA Library

RNA was extracted from Cf9 tobacco cells 30 min after adding IF(Avr9$^+$). Poly(A) RNA was isolated from 1.25 mg on oligo(dT) cellulose columns (Pharmacia). cDNA was synthesized and inserted into the Uni-ZAP XR λ insertion vector (Stratagene, La Jolla, CA). After packaging (Gigapack III Gold; Stratagene), the library was plated at a density of 50,000 plaques per 20 × 20-cm plate. Plaque lifts were screened with the reamplified AFLP fragments, which were radiolabeled with ^{32}P-dCTP using an oligolabeling kit (Pharmacia). Hybridization was performed following the protocol of Church and Gilbert (1984). Filters were washed four times for 15 min with 100 mL of 1% SDS and 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate) at 65°C.

RNA Gel Blot Analysis

Aliquots (5 to 10 μg) of total RNA were separated by electrophoresis on a denaturing 1.4% agarose gel (100 mL of denaturing gel contained 1.4 g of agarose and 5.4 mL of 12.3 M formaldehyde [37% w/w]) followed by blotting onto nylon membrane (Hybond-N; Amersham, UK) (Sambrook et al., 1989). The RNA was cross-linked to the membrane using a UV Stratalinker (model 2400; Stratagene). Probe labeling and hybridization were performed as given above. The membranes were washed with 0.1% SDS and 2 × SSC twice for 10 min and subsequently with 0.1% SDS and 0.5 × SSC twice for 30 min at 65°C.

Isolation of Hin1, Hsr203J, and EREBP1 cDNAs from Tobacco

The Hin1, Hsr203J, and EREBP1 cDNAs were amplified from tobacco cDNA by PCR using specific primers. PCR products were ligated into the pGEM-T EASY vector (Promega, Southampton, UK), and the identity of the clones was verified by sequencing. Clone inserts were used as probes on RNA gel blots. For the EREBP1 probe, a 239-bp C-terminal fragment was used. This fragment did not contain the DNA binding domain.

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