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Acquired Resistance in Arabidopsis

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Acquired resistance is an important component of the complex disease resistance mechanism in plants, which can result from either pathogen infection or treatment with synthetic, resistance-inducing compounds. In this study, Arabidopsis, a tractable genetic system, is shown to develop resistance to a bacterial and a fungal pathogen following 2,6-dichloroisonicotinic acid (INA) treatment. Three proteins that accumulated to high levels in the apoplast in response to INA treatment were purified and characterized. Expression of the genes corresponding to these proteins was induced by INA, pathogen infection, and salicylic acid, the latter being a putative endogenous signal for acquired resistance. Arabidopsis should serve as a genetic model for studies of this type of immune response in plants.

INTRODUCTION

Immunization or acquired resistance in plants has been documented for more than 50 years (Chester, 1933). In contrast to specific, heritable resistance (Keen, 1990), acquired resistance must be triggered by a pathogen infection that causes necrosis in the inoculated tissue. Once induced, a plant is resistant to a wide range of different pathogens (Ryals et al., 1992). In addition to biotic inducers, certain chemicals with no direct antibiotic effect can also induce resistance in plants. These include natural products such as salicylic acid (SA) (White, 1979) and synthetic immunomodulators such as 2,6-dichloroisonicotinic acid (INA) (Métraux et al., 1991).

Acquired resistance has been characterized best in tobacco and cucumber (Kuc, 1982; Ward et al., 1991b). In the early 1960s, A. F. Ross demonstrated that infection with tobacco mosaic virus can cause tobacco to become resistant to diverse viral pathogens (Ross, 1961a, 1961b, 1966). This phenomenon, also termed systemic acquired resistance (SAR), was shown to be effective against fungal and bacterial pathogens as well (Hecht and Bateman, 1964; Kuc, 1982). Recently, the development of SAR has been closely correlated with the expression of a set of nine gene families called SAR genes (Ward et al., 1991b). Five of these gene families encode the pathogenesis-related (PR) proteins, a set of extracellular polypeptides long associated with pathogen infection (for review, see Bol et al., 1990). The physiological functions of the PR proteins have not yet been established. Some, however, belong to protein classes (chitinases, β-1,3-glucanases, and thaumatin-like proteins) with demonstrated in vitro antifungal activity, alone or in combination (Mauch et al., 1988; Roberts and Selitrennikoff, 1988; Vigers et al., 1991; Woloshuk et al., 1991). These observations suggest that the SAR proteins may play a causal role in the immunized state.

Although this resistant state is now relatively well characterized in tobacco, little is known about the sequence of events occurring between the inducing stimulus and the onset of resistance. The synthesis and release of a diffusible signal compound from the site of the pathogen-induced necrotic lesion that moves through the phloem has been postulated (Ross, 1966; Jenns and Kuc, 1977). Several lines of experimental evidence now indicate that SA could be such an endogenous signal in both tobacco and cucumber. Specifically, SA levels in the phloem dramatically increase following pathogen infection of cucumber and tobacco (Malamy et al., 1990; Métraux et al., 1990). This increase in endogenous SA is sufficient to induce PR proteins (Yalpani et al., 1991), and exogenously applied SA has been shown to induce both SAR gene expression and resistance (White, 1979; Ward et al., 1991b). Although these observations provide compelling evidence that SA plays a role in signaling acquired resistance, other experiments suggest that another signal may be present that translocates through the plant and induces the synthesis or release of SA (Rasmussen et al., 1991).

One approach to understanding the biochemical changes leading to acquired resistance is the analysis of mutants defective in signal transduction. Because the organisms in which acquired resistance has been characterized are relatively poor genetic systems, we sought to determine whether Arabidopsis possesses an acquired resistance response. This crucifer has become a model genetic system for higher plants due to its rapid generation time and small, well-mapped genome (for

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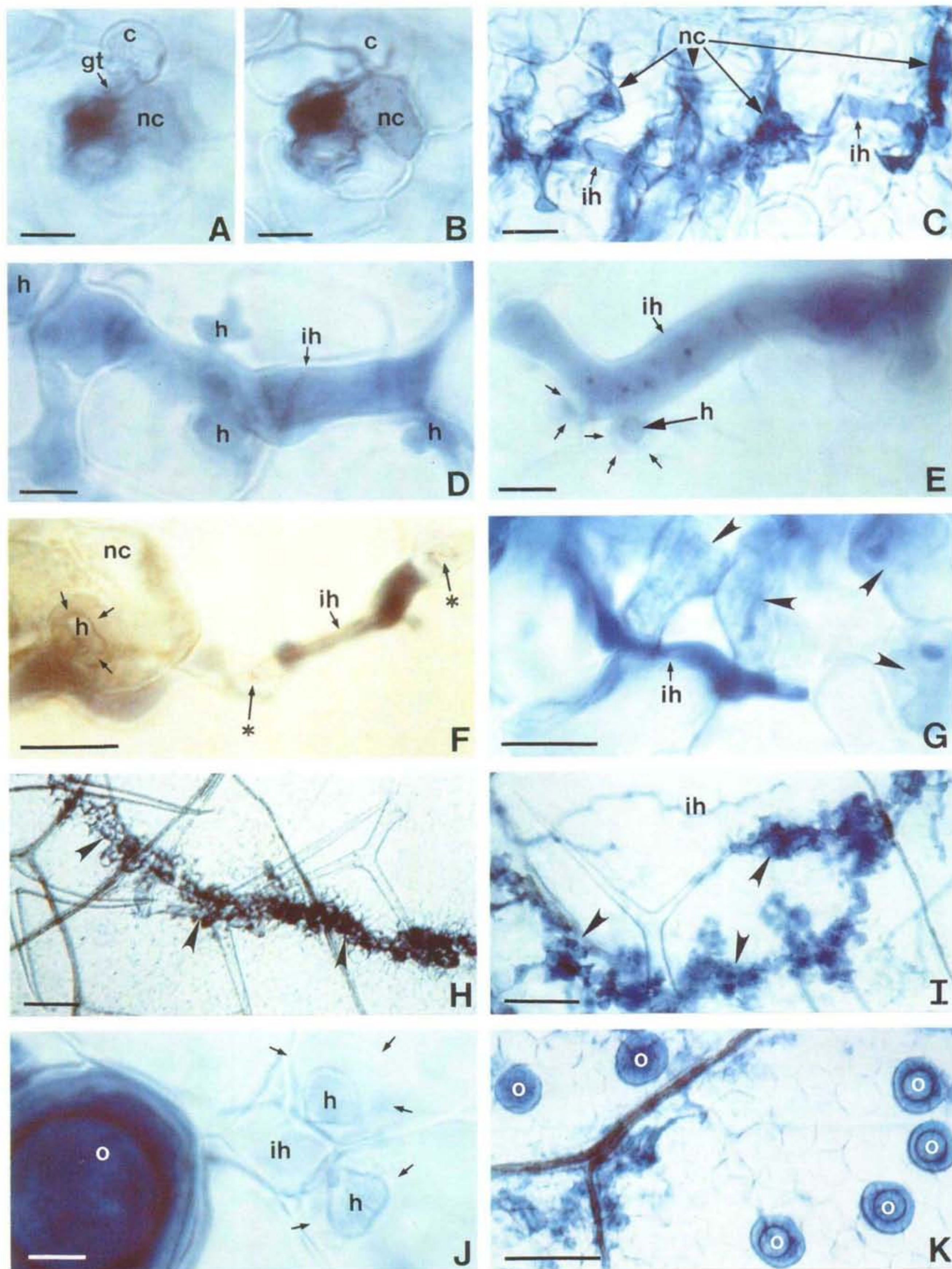

Figure 1. Cytology of the Infection of INA-Treated Leaves by *P. parasitica*.

review, see Meyerowitz, 1989). Here, we characterize biological and molecular aspects of chemically mediated acquired resistance in Arabidopsis.

RESULTS

Biological Responses of Arabidopsis to Chemical Immunization

Plants were treated with varying concentrations of INA and, at increasing intervals after treatment, were inoculated with an isolate of *Peronospora parasitica* that causes downy mildew of Arabidopsis (Koch and Slusarenko, 1990). Trypan blue staining was used to visualize the plant–fungus interaction because it penetrates fungal cells easily and preferentially stains plant cells that have suffered membrane damage (Keogh et al., 1980). When untreated or mock treated Arabidopsis (accession Weiningen or ecotype Landsberg *erecta*) was infected with a spore suspension of *P. parasitica*, a typical compatible biotrophic interaction was observed. This was evident at the macroscopic level as the formation of a lawn of conidiophores on the leaf surface (Koch and Slusarenko, 1990). At the microscopic level, intercellular hyphae and intracellular haustoria and oospores were observed. Plant cell necrosis was not evident until the late stages of the infection (Koch and Slusarenko, 1990).

Table 1 shows that soil treatment of Arabidopsis plants with INA concentrations as low as 0.52 μM (0.1 ppm) induced significant resistance to *P. parasitica* infection. The highest concentration used, 52 μM, completely inhibited hyphal growth. Figure 1 illustrates the spectrum of responses of the plant–fungus interaction to INA treatment. At higher INA concentrations, both Weiningen and Landsberg reacted with predominantly single-celled necroses at sites of attempted penetration by the normally virulent *P. parasitica* isolate (Figures 1A and 1B). In general, hyphae did not penetrate beyond these initial infection sites, but in the few cases where penetration was observed, hyphae became surrounded by a cluster of necrotic plant cells and growth ceased (Figure 1C). Growth

Table 1. Macroscopic Asexual Sporulation Rating

Treatment	Ecotype	Time from Treatment to Inoculation		
		Day 1	Day 4	Day 7
None	Weiningen[a]	+ + + + +	+ + + + +	+ + + + +
	Landsberg[b]	+ + + +	+ + + +	+ + + +
WP	Weiningen	+ + + + +	+ + + + +	+ + + + +
	Landsberg	+ + + +	+ + + +	+ + + +
0.52 μM INA	Weiningen	NT	NT	NT
	Landsberg	+ + + +	–	–
5.2 μM INA	Weiningen	+ +	–	–
	Landsberg	–	–	–
52 μM INA	Weiningen	+	–	–
	Landsberg	–	–	–

[a] Weiningen was scored 8 days after inoculation.
[b] Landsberg *erecta* was scored 11 days after inoculation.
WP, wettable powder carrier without INA; –, no sporulation; + to + + + + +, increasing degrees of sporulation; NT, not tested.

Figure 1. (continued).

(A) and **(B)** Different focal planes of the same preparation of a leaf of Arabidopsis ecotype Landsberg *erecta* treated with INA (52 μM) 1 day before inoculation with *P. parasitica*. Necrotic epidermal cell (nc) and guard cells adjacent to a germinating conidium are shown. c, conidium; gt, germ tube. Bar = 25 μm.
(C) Necrotic cells framing an intercellular hypha (ih) of *P. parasitica* in a leaf of Landsberg *erecta* from a plant treated with INA (5.2 μM) 7 days before inoculation. Bar = 25 μm.
(D) Intercellular hypha and haustoria (h) of *P. parasitica* in a leaf of an untreated control plant of Landsberg *erecta* 7 days after inoculation. Bar = 10 μm.
(E) Intercellular hypha with encased (arrows) haustoria in a leaf of Arabidopsis accession Weiningen treated with INA (5.2 μM) 4 days before inoculation. Bar = 10 μm.
(F) *P. parasitica* in a leaf of Landsberg *erecta* treated with INA (0.52 μM) 4 days before inoculation. The intercellular hypha is partially lacking cytoplasm (*), especially at the hyphal tip. An encased (arrows) haustorium is located in a necrotic cell. Bar = 25 μm.
(G) Necrotic cells (arrowheads) surrounding an intercellular hypha of *P. parasitica* in a leaf of Landsberg *erecta* treated with 5.2 μM INA 4 days before inoculation. Bar = 25 μm.
(H) A trail of necrotic cells (arrowheads) reveals the path of a hypha of *P. parasitica* in a leaf of Landsberg *erecta* treated with 52 μM INA 7 days before inoculation. Bar = 100 μm.
(I) A trail of necrotic cells (arrowheads) and a weak-looking intercellular hyphae of *P. parasitica* in a leaf of Weiningen treated with 5.2 μM INA 4 days before inoculation. Bar = 100 μm.
(J) Oospore (o) of *P. parasitica* in an untreated control leaf of Landsberg *erecta* 11 days after inoculation. At the time of oospore formation, haustoria, especially in the older parts of the mycelium, became encased (arrows). Bar = 11 μm.
(K) Oospores in a leaf of an untreated plant of Landsberg *erecta* 7 days after inoculation. Clusters of trypan blue–stained necrotic cells can be seen, but the fungal mycelium is no longer distinct. Bar = 100 μm.
Plates in **(A)** and **(B)** were photographed 1 day after inoculation; plates in **(C)**, and **(E)** to **(H)** were photographed 3 days after inoculation; plates in **(D)**, **(I)**, and **(K)** were photographed 7 days after inoculation; and plate in **(J)** was photographed 11 days after inoculation.

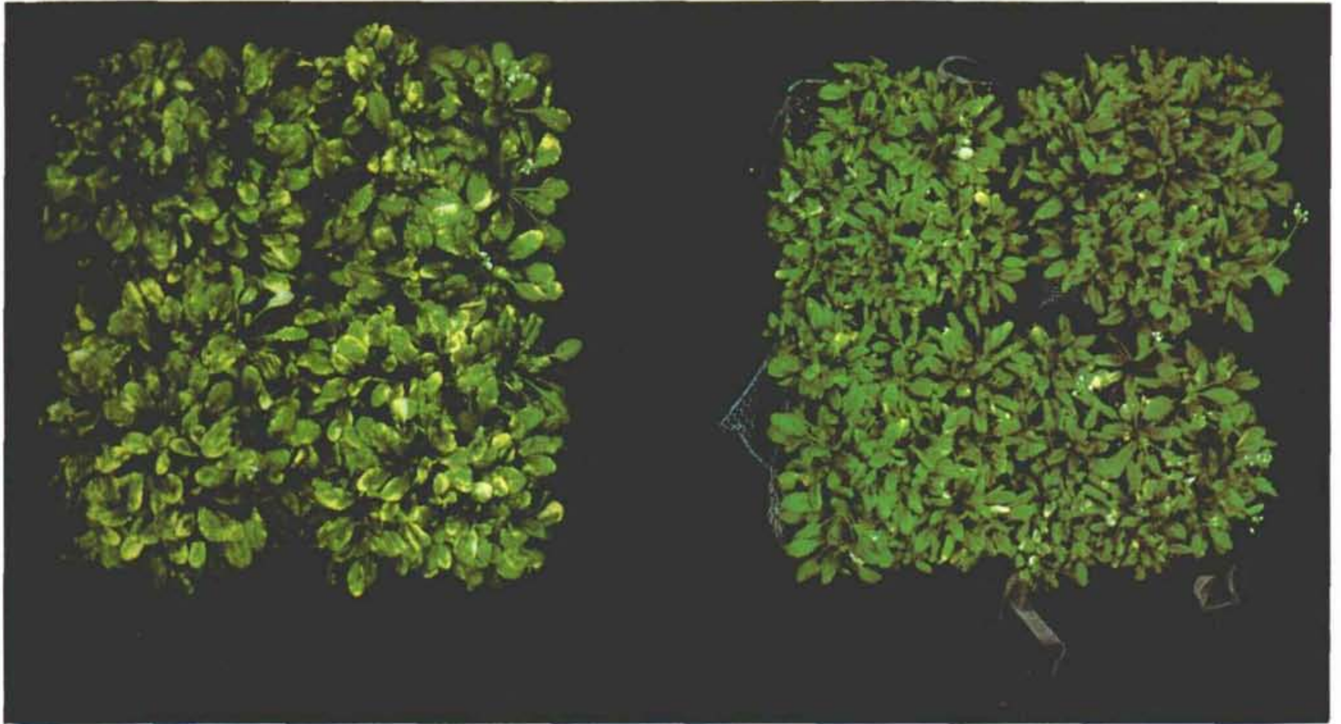

Figure 2. INA-Induced Resistance against Infection by *P.s. tomato* DC3000.

Plants were treated with either water **(left)** or INA **(right)** and subsequently infected with *P. s. tomato* DC3000. The photograph was taken 4 days after infection.

of hyphae in untreated control plants is shown in Figure 1D for comparison. Note the absence of both plant cell necrosis and encasements around the haustoria.

Fungus growing on plants treated with lower concentrations of INA displayed significantly reduced asexual sporulation depending on the time from pretreatment to infection (Table 1). Figure 1E shows the proximal portion of an intercellular hypha with haustoria reduced in size and encased in material presumably of host origin. Such encasements have been demonstrated to contain callose (B. Mauch-Mani, unpublished results). Although haustorial encasements are observed in a compatible interaction, they normally occur adjacent to older parts of the mycelium and never near the hyphal tips (Koch and Slusarenko, 1990). Portions of the mycelium in INA-treated plants often lacked cytoplasm; this effect seemed to be most pronounced at the hyphal tip (Figure 1F). Occasionally, INA-treated cells containing encased haustoria became necrotic (Figure 1F), which was never observed in control plants. Sparse colonization of INA-treated leaf tissue by the fungus was sometimes observed but was always followed closely by host cell death. In such cases, the plant cells seemed to die trailing in the wake of the growing hypha until hyphal growth ceased altogether (Figures 1G to 1I). Hyphae from INA-treated plants were thinner than in control plants and stained less intensely with trypan blue (Figure 1I). In contrast, in a normal compatible interaction, only haustoria from older parts of the mycelium became encased, and these encasements typically occurred in living cells (Figure 1J). Moreover, host cell necrosis was not evident until the time of oospore formation, and the association of necrotic cells with the presence of the fungus was not clear (Figure 1K).

INA-induced resistance was also observed in plants infected with *Pseudomonas syringae* pv *tomato* DC3000, which causes bacterial speck disease of tomato and which is virulent on Arabidopsis ecotype Columbia (Whalen et al., 1991). Plants were treated by spraying foliage with INA at 0.65 mM; these plants were then infected 9 days later by dipping in a suspension of *P.s. tomato.* Figure 2 shows symptom development in INA-treated plants compared with water-treated controls. The controls displayed small grayish-brown lesions, surrounded by extensive spreading chlorosis, typical of the interaction between *P.s. tomato* DC3000 and ecotype Columbia (Whalen et al., 1991). INA-treated plants showed dramatically fewer lesions and chlorosis. Foliar treatment with this concentration of INA caused a mild phytotoxic effect manifested as decreased plant size, narrower leaf shape, and occasional chlorosis only at the leaf margin. A lower concentration of INA (65 μM) induced a similar level of resistance but did not cause phytotoxicity (see cover). To determine whether decreased symptom development was linked with reduction in bacterial population, *P.s. tomato* cell number was assayed in leaves of control and INA-treated plants. At least 10-fold more bacteria were found in control plants than in INA-treated plants, as illustrated in Figure 3.

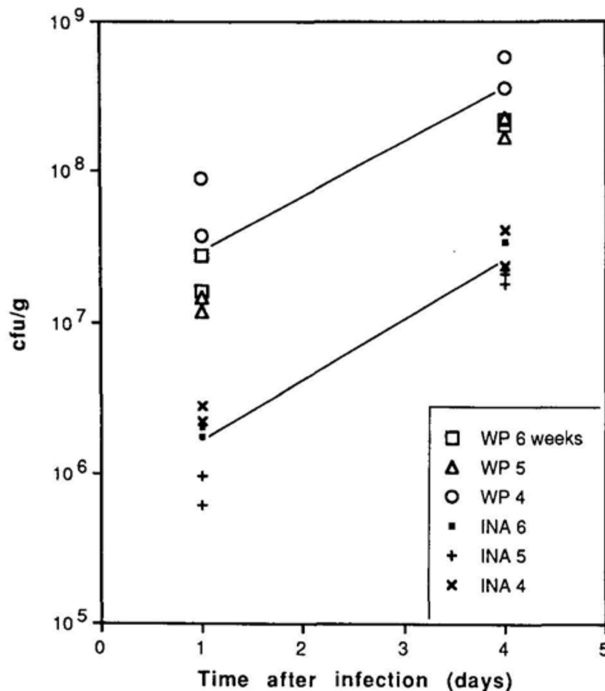

Figure 3. Determination of Bacterial Titer in Control and INA-Treated Arabidopsis.

Plants of three different ages (4, 5, and 6 weeks) were either mock treated with wettable powder (WP) or chemically treated (INA) and subsequently infected with *P.s. tomato* DC3000. Each point represents a pooled extract from at least 10 leaves. Lines connect the means of the WP and INA treatments. cfu/g, rifampicin-resistant colony forming units per gram fresh weight of leaf tissue.

Biochemical Responses of Arabidopsis to Chemical Treatment

In tobacco, INA and other immunizing agents induce the dramatic accumulation of mRNAs that encode a variety of extracellular proteins (Ward et al., 1991b). Using PAGE, we examined proteins present in the intercellular wash fluid (ICF) of Arabidopsis plants that had been treated with INA or with water as a control. Figure 4 shows separation of these proteins under denaturing and native conditions. Four new proteins, migrating at 10, 16, 26, and 37 kD, were observed in the ICF from INA-treated tissue when analyzed by denaturing PAGE (Figure 4A). The 26- and 37-kD proteins were gel purified by native PAGE (Figure 4B). The 16-kD protein was purified by reverse phase HPLC and analyzed on denaturing polyacrylamide gels (Figure 4C). Each protein was digested to yield peptides, and their partial amino acid sequences were determined. By comparing these sequences to a data base of pathogen-induced proteins (M. Moyer, unpublished results), it was clear that the 16-kD protein was similar to the PR-1 proteins of tobacco, the 26-kD protein was similar to the PR-5 (thaumatin-like) proteins of tobacco, and the 37-kD protein was related to the PR-2 (β-1,3-glucanase) protein family of tobacco. The 10-kD band gave rise to peptides with sequences identical to the C-terminal third of the PR-2 protein and was apparently a breakdown product. The diffuse protein band migrating at approximately 42 kD (Figure 4A) was not reproducibly induced.

cDNA Clones Corresponding to the Induced Proteins

cDNA clones for the three extracellular proteins were isolated from a library made from chemically induced leaf tissue. The PR-1–related cDNA was isolated by cross-hybridization to tobacco PR-1 acidic and basic cDNAs at low stringency (Payne et al., 1988b, 1989). The sequence of the longest of three identical clones is shown in Figure 5A. Figure 6A shows that the predicted translation product matched exactly the sequenced peptides from the 16-kD extracellular protein, which we designate Arabidopsis PR-1. The predicted translation product had 161 amino acid residues and a molecular weight of 17677. The protein was approximately 60% identical to both the acidic and basic forms of PR-1 from tobacco (Figure 6A). Based on comparison to the sequences of the tobacco heterologs, the primary translation product is a preprotein that is cleaved to yield a mature polypeptide of 135 residues, with a molecular weight of 14880 and a calculated pI of 8.5. These values are consistent with the behavior of the protein during electrophoresis.

PR-2 cDNAs were isolated from the library with a degenerate oligonucleotide probe corresponding to the amino acid sequence of one of its peptides. Four clones were isolated that were identical over their predicted coding sequence. The sequence of the longest clone is shown in Figure 5B. The translated open reading frame of 305 codons matched the sequenced peptides from the 37-kD protein. Comparison of this Arabidopsis PR-2 protein to related proteins from tobacco predicted a start of the mature polypeptide at residue 11, yielding a processed molecular weight of 32422 and pI of 4.8. The Arabidopsis PR-2 protein was 56% identical to the PR-Q′ glucanase from tobacco, 51% identical to the acidic PR-2 glucanase, and 52% identical to the basic β-1,3-glucanase Figure 6B).

A cDNA encoding the PR-5 (thaumatin-like) protein was amplified by the polymerase chain reaction (Saiki et al., 1988) from double-stranded cDNA using degenerate primers corresponding to portions of the peptide sequences. A product of the predicted size was isolated and used as a probe to screen the cDNA library. Five isolates were analyzed, and one containing a full-length coding sequence was sequenced (Figure 5C). The predicted protein product was 238 residues in length and matched the peptide sequences at all positions (Figure 6C). The open reading frame extended 23 codons upstream of the N-terminal residue determined by protein sequencing, which is consistent with the presence of a signal peptide in the primary translation product. The mature protein, designated Arabidopsis PR-5, had a predicted molecular weight of 22724

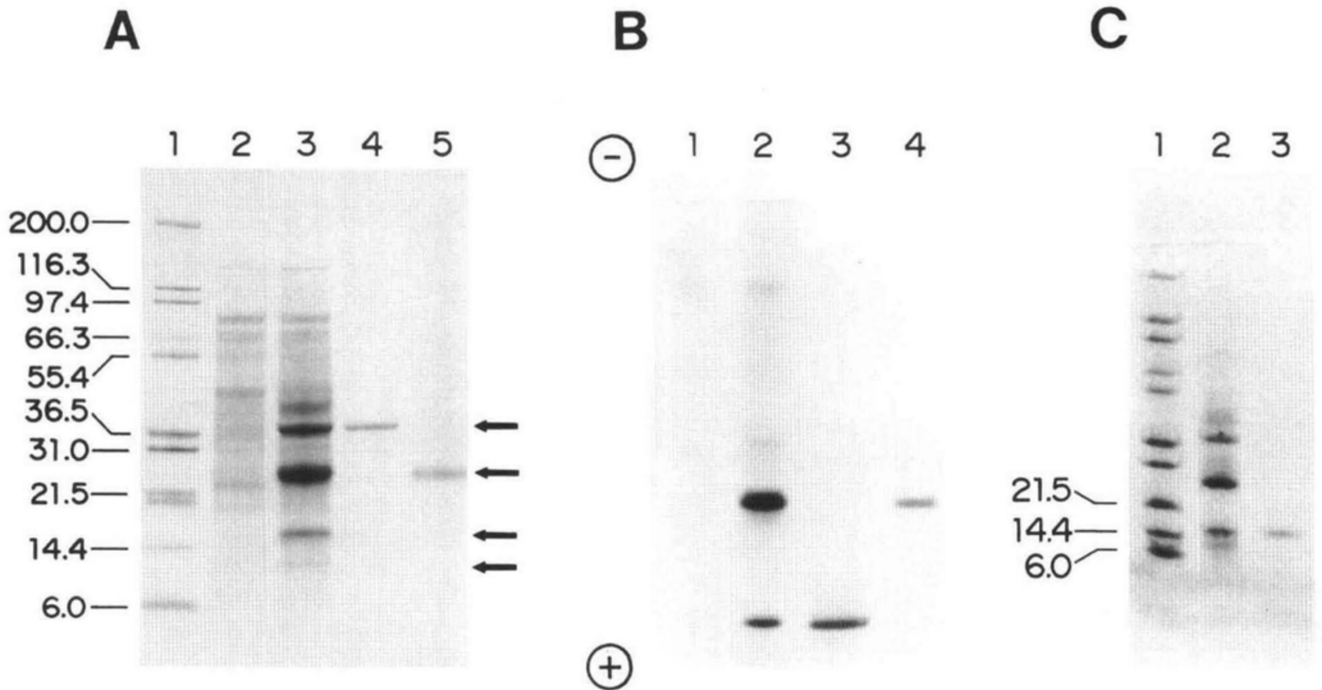

Figure 4. Extracellular Proteins Induced in Response to INA Treatment.

(A) SDS–polyacrylamide gel. Lane 1, molecular mass markers given in kD; lane 2, total ICF proteins from water-treated control plants; lane 3, total ICF proteins from INA-treated plants; lane 4, the purified PR-2 protein (β-1,3-glucanase); lane 5, the purified PR-5 protein (thaumatin-like). Arrows indicate the positions of proteins that were purified.
(B) Native polyacrylamide gel, used for purification of the PR-2 and PR-5 proteins. Lane 1, total ICF proteins from water-treated plants; lane 2, total ICF proteins from INA-treated plants; lane 3, the purified PR-2 protein; lane 4, the purified PR-5 protein. +, the anodal end of the gel; −, the cathode.
(C) Purification of the PR-1 protein, shown on an SDS–polyacrylamide Phast gel (Pharmacia). Lane 1, molecular mass markers; lane 2, total ICF protein from INA-treated plants. The PR-1 protein is clearly resolved as the upper band of a doublet not resolved in the gel system shown in **(A)**. Lane 3, the purified PR-1 protein.
The gels were stained with Coomassie Brilliant Blue R250.

and a pI of 4.5, in agreement with its relative position on SDS and native polyacrylamide gels. The Arabidopsis PR-5 protein was approximately 46% identical to the acidic and basic PR-5 proteins of tobacco (Figure 6C). All 16 cysteines were conserved between the Arabidopsis PR-5 and other thaumatin-like proteins.

Gene Expression Associated with Acquired Resistance

Expression of the genes corresponding to the cDNAs was examined in Arabidopsis plants treated with several agents known to induce resistance. Plants were treated with INA by foliar spray or root drench application and harvested at various days after treatment. Gel blots of RNA samples were probed with each of the three cDNAs. Figure 7A shows the results of a typical foliar time course experiment. Within 1 day of INA treatment, PR-1 mRNA abundance was induced more than 50-fold, and PR-2 and PR-5 mRNAs were induced approximately 20-fold. Gene expression in response to INA spray treatment appeared identical in Arabidopsis ecotypes Columbia and Landsberg and accession Weiningen (S. Uknes and S. Potter, unpublished data). In plants treated with INA by soil drench, expression of the three genes increased from low background levels in a dose- and time-dependent fashion (data not shown).

To determine whether SA would induce expression of these genes, plants were sprayed with 5 mM SA and analyzed as for the INA time course experiments (Figure 7B). The RNAs accumulated to high levels at the earliest time point examined, showing greater than 20-fold induction within 1 day after SA treatment. However, unlike INA treatment, SA-induced expression decreased at later time points. The significance of this observation is unclear but may relate to differences between INA and SA metabolism in plants.

To test whether these genes were inducible by pathogen infection, leaves were infected with *P.s. tomato*, then harvested at 6 days after infection. RNA levels were analyzed by gel blot hybridizations. Expression of each of the three genes was

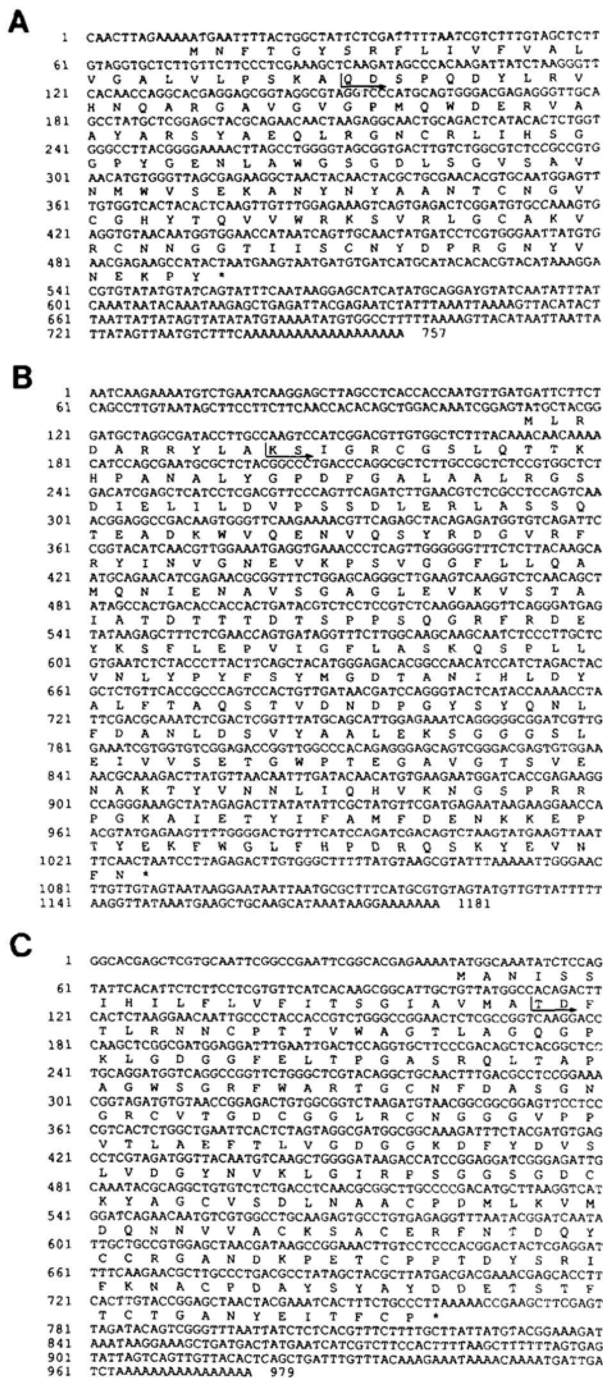

Figure 5. Sequence of the cDNAs Encoding INA-Induced Proteins.

(A) PR-1.
(B) PR-2.
(C) PR-5.

The N terminus of the mature protein is indicated by an arrow under the translated sequence. For the PR-1–like protein and the PR-2 protein, the presumptive N termini were identified by comparison to tobacco proteins of the same classes. GenBank accession numbers are M90508 for PR-1, M90509 for PR-2, and M90510 for PR-5.

strongly induced by pathogen infection (Figure 7C). PR-1 and PR-2 were induced more than 20-fold and PR-5 was induced more than 10-fold by *P.s. tomato* infection.

DISCUSSION

Chemical treatment caused Arabidopsis to respond dramatically to *P. parasitica* infection. Single-celled necroses at the attempted penetration sites in tissues treated with high concentrations of INA (Figures 1A and 1B) are reminiscent of the hypersensitive reaction, which occurs in a genetically incompatible plant–pathogen interaction (Koch and Slusarenko, 1990). However, the mechanism of cell death in this case may be different from a bona fide hypersensitive reaction resulting from genetic incompatibility. The trailing necrosis that follows growing hyphae in some plants treated with INA (Figures 1H and 1I) is not typical of genetic incompatibility but is similar to the effects of a subclinical dose of fungicide on a compatible plant–fungus interaction (Király et al., 1972).

Two lines of evidence suggest that the fungicidal response observed in INA immunization results not from direct effects on the pathogen but from a reaction of the plant to the chemical. First, neither INA nor its metabolites have direct antibiotic activity (Métraux et al., 1991). Second, INA has been shown to cause dramatic changes in plant gene expression that are similar to the changes observed during pathogen-induced immunization (Ward et al., 1991b). Thus, INA may induce resistance by mimicking some aspects of pathogen attack, possibly accelerating the normal responses of the plant to further infection.

INA-mediated acquired resistance in Arabidopsis was not specific to a given pathogen, as manifested by its effectiveness with *P.s. tomato* infection. The INA-conditioned decrease in symptom formation with *P.s. tomato* infection correlated with a 10-fold decrease in bacterial titer in leaf tissue. Therefore, the treated plants seem to exert an antibacterial effect, analogous to the apparently fungicidal response observed in the *P. parasitica* interaction.

The reduced pathogenesis observed in INA-treated tissues correlated well with the accumulation of PR-1, PR-2, and PR-5 mRNAs and proteins. PR-2 is structurally similar to a β-1,3-glucanase, a type of protein known to enhance the in vitro antifungal activity of chitinases (Mauch et al., 1988). PR-5 is closely related to the tobacco protein osmotin and the maize seed protein zeamatin, both of which have in vitro antifungal activity (Vigers et al., 1991; Woloshuk et al., 1991). In tissues treated with lower INA concentrations, effects on *P. parasitica* hyphal morphology (for example, cleared tips and spindly growth) were similar to changes observed when mycelia of other fungi were treated in vitro with a combination of β-1,3-glucanase and chitinase or zeamatin (Mauch et al., 1988; Roberts and Selitrennikoff, 1988, 1990). Thus, it is tempting to speculate that expression of the Arabidopsis PR proteins is at least partially responsible for the resistance observed.

A

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Peptides                                                      GAVGV
Arab.PR-1    MNFTGYSR.. .FLIVFVALV GALVL.PSKA QDSPQDYLRV HNQARGAVGV
Tob.PR-1a    MGFVLFSQLP SFLLVSTLLL FLVISHSCRA QNSQQDYLDA HNTARADVGV
Tob.PR-1b    MGFFLFSQMP SFFLVSTLLL FLIISHSSHA QNSQQDYLDA HNTARADVGV
Tob.PR-1c    MEFVLFSQMS SFFLVSTLLL FLIISHSCHA QNSQQDYLDA HNTARADVGV
Tob.basic    MGFLTT.... ...IVACFIT FAILIHSSKA QNSPQDYLNP HNAARRQVGV

Peptides     GPMQWDERVA AYARSYAEQL R
Arab.PR-1    GPMQWDERVA AYARSYAEQL RGNCRLIHSG GPYGENLAWG SGD.LSGVSA
Tob.PR-1a    EPLTWDDQVA AYAQNYASQL AADCNLVHSH GQYGENLAEG SGDFMTAAKA
Tob.PR-1b    EPLTWDNGVA AYAQNYVSQL AADCNLVHSH GQYGENLAQG SGDFMTAAKA
Tob.PR-1c    EPLTWDDQVA AYAQNYASQL AADCNLVHSH GQYGENLAWG SGDFLTAAKA
Tob.basic    GPMTWDNRLA AYAQNYANQR IGDCGMIHSH GPYGENLAAA FP.QLNAAGA

Peptides             AN YNYAANT
Arab.PR-1    VNMWVSEKAN YNYAANTC.. NGVCGHYTQV VWRKSVRLGC AKVRCNNGGT
Tob.PR-1a    VEMWVDEKQY YDHDSNTCAQ GQVCGHYTQV VWRNSVRVGC ARVQCNNGGY
Tob.PR-1b    VEMWVDEKQY YDHDSNTCAQ GQVCGHYTQV VWRNSVRVGC ARVKCNNGGY
Tob.PR-1c    VEMWVNEKQY YAHDSNTCAQ GQVCGHYTQV VWRNSVRVGC ARVQCNNGGY
Tob.basic    VKMWVDEKRF YDYNSNSCVG G.VCGHYTQV VWRNSVRLGC ARVRSNNGWF

Peptides              G NYVNEKPY
Arab.PR-1    IISCNYDPRG NYVNEKPY*
Tob.PR-1a    VVSCNYDPPG NYRGESPY*
Tob.PR-1b    VVSCNYDPPG NVIGQSPY*
Tob.PR-1c    IVSCNYDPPG NVIGKSPY*
Tob.basic    FITCNYDPPG NFIGQRPFGD LEEQPFDSKL ELPTDV*
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B

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Arab.PR-2    M......... .......... ...LRDARRY LAKSIGRCGS LQTTKHPANA
Tob.acidic   M...TLCIKN GFLAAALVLV GLLICSIQMI GAQSIGVCYG KHANNLPSDQ
Tob.basic    MSTSHKHNTP QMAAITLLGL LLVASSIDIA GAQSIGVCYG MLGNNLPNHW
Tob.PR-Q'    M........A HLIVTLLLLS VLTLATLDFT GAQA.GVCYG RQGNGLPSPA

Arab.PR-2    .......... .....LYGPD PGALAALRGS DIELILDVPS SDLERLASSQ
Tob.acidic   DVINLYDANG IRKMRIYNPD TNVFNALRGS NIEIILDVPL QDLQSLTDP.
Tob.basic    EVIQLYKSRN IGRLRLYDPN HGALQALKGS NIEVMLGLPN SDVKHIASGM
Tob.PR-Q'    DVVSLCNRNN IRRMRIYDPD QPTLEALRGS NIELMLGVPN PDLENVAASQ

Arab.PR-2    TEADKWVQEN VQSYRDGVRF RYINVGNEVK PSVG.....G FLLQAMQNIE
Tob.acidic   SRANGWVQDN IINHFPDVKF KYIAVGNEVS P.GNNGQYAP FVAPAMQNVY
Tob.basic    EHARWWVQKN VKDFWPDVKI KYIAVGNEIS PVTGTSYLTS FLTPAMVNIY
Tob.PR-Q'    ANADTWVQNN VRNY.GNVKF RYIAVGNEVS PLNENSKYVP VLLNAMRNIQ

Peptides        VSTAIAT DTTTDTSPPS QGR      S FLEPVIGFLA
Arab.PR-2    NAVSGAGL.. EVKVSTAIAT DTTTDTSPPS QGRFRDEYKS FLEPVIGFLA
Tob.acidic   NALAAAGLQD QIKVSTATYS GILANTNPPK DSIFRGEFNS FINPIIQFLV
Tob.basic    KAIGEAGLGN NIKVSTSVDM TLIGNSYPPS QGSFRNDARW FVDPIVGFLR
Tob.PR-Q'    TAISGAGLGN QIKVSTAIET GLTTDTSPPS NGRFKDDVRQ FIEPIINFLV

Peptides     S
Arab.PR-2    SKQSPLLVNL YPYFSYMGDT ANIHLDYALF TAQSTVDNDP GYSYQNLFDA
Tob.acidic   QHNLPLLANV YPYFGHIFNT ADVPLSYALF TQQE....AN PAGYQNLFDA
Tob.basic    DTRAPLLVNI YPYFSYSGNP GQISLPYSLF TAPNVVVQDG SRQYRNLFDA
Tob.PR-Q'    TNRAPLLVNL YPYFAIANN. ADIKLEYALF TSSEVVVNDN GRGYRNLFDA

Peptides                SGGGSLEIV VSETG?PTE            TYVNNLIQH
Arab.PR-2    NLDSVYAALE KSGGGSLEIV VSETGWPTEG AVG.TSVENA KTYVNNLIQH
Tob.acidic   LLDSMYFAVE KAGGQNVEII VSESGWPSEG NSAAT.IENA QTYYENLINH
Tob.basic    MLDSVYAALE RSGGASVGIV VSESGWPSAG AFGAT.YDNA ATYLRNLIQH
Tob.PR-Q'    ILDATYSALE KASGSSLEIV VSESGWPSAG AGQLTSIDNA RTYNNNLISH

Peptides     VK            AIETYIF AMFDENK           FWGLF HPDRQSK
Arab.PR-2    VKNGS..PRR PGKAIETYIF AMFDENKKEP TY.EKFWGLF HPDRQSKYEV
Tob.acidic   VKSGAGTPKK PGNAIETYLF AMFDENNKEG DITEKHFGLF SPDQRAKYQL
Tob.basic    AKEGS..PRK P.GPIETYIF AMFDENNKNP EL.EKHFGLF SPNKQPKYNI
Tob.PR-Q'    VKGGS..PKR PSGPIETYVF ALFDEDQKDP EI.EKHFGLF SANMQPKYQI

Arab.PR-2    NFN*
Tob.acidic   NFN*
Tob.basic    NFGVSGGVWD SSVETNATAS LVSEM*
Tob.PR-Q'    SFN*
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C

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Peptides                             TDFTL RNNCT TVWA GTLAGNGPK
Arab.PR-5      MANISSIHIL FLVFITSGIA VM..ATDFTL RNNCPTTVWA GTLAGQGPKL
Tob.PR-5maj.   MNFLKSFPFF AFLYFGQYFV AVTHAATFDI VNKCTYTVWA AASP......
Tob.PR-5min.   MNFLKSFPFY AFLCFGQYFV AVTHAATFDI VNQCTYTVWA AASPG.....
Osmotin        MGNLRSSFVF FLLALVTYTY ....AATIEV RNNCPYTVWA ASTPI.....

Arab.PR-5      GDGGFELTPG ASRQLTAPAG WS....... .GRFWARTGC NFDASGNGRC
Tob.PR-5maj.   .........G GGRRLDSGQS WSINVNPGTV QARIWGRTNC NFDGSGRGNC
Tob.PR-5min.   .......... GGRQLNSGQS WSINVNPGTV QARIWGRTNC NFDGSGRGNC
Osmotin        .........G GGRRLDRGQT WVINAPRGTK MARVWGRTNC NFNAAGRGTC

Peptides                                          KDFYDVSL VDGYNVK
Arab.PR-5      VTGDCGG.LR CNGGGVPPVT LAEFTLVGDG .GKDFYDVSL VDGYNVKLGI
Tob.PR-5maj.   ETGDCNGMLE CQGYGKAPNT LAEFALN.QP N.QDFVDISL VDGFNIPMEF
Tob.PR-5min.   ETGDCNGMLE CQGYGKPPNT LAEFALN.QP N.QDFVDISL VDGFNIPMEF
Osmotin        QTGDCGGVLQ CTGWGKPPNT LAEYALD.QF SGLDFWDISL LDGFNIPITF

Peptides                     YAGCVSD LNAACPDMLK            SACERFNTD
Arab.PR-5      RP...SGGSG DCKYAGCVSD LNAACPDMLK VMDQNNVVAC KSACERFNTD
Tob.PR-5maj.   .PTN..GG.. .CRNLRCTAP INEQCPAQLK TQG.....GC NNPCTVIKTN
Tob.PR-5min.   SPTN  GG.. .CRNLRCTAP INEQCPAQLK TQG.....GC NNPCTVIKTN
Osmotin        .PTNPSGG.. KCHAL CTA  INGECPAELR VPG.....GC NNPCTTFGGQ

Peptides       QYCCRGANDK PETCPPTDYS RIFK
Arab.PR-5      QYCCRGANDK PETCPPTDYS RIFKNACPDA YSYAYDDETS TFTC.TGA.N
Tob.PR-5maj.   EYCCTNG... PGSCGPTDLS RFFKERCPDA YSYPQDDPTS LFTCPSGT.N
Tob.PR-5min.   EFCCTNG... PGSCGPTDLS RFFKARCPDA YSYPQDDPPS LFTCPPGT.N
Osmotin        QYCCT..... QRPCGPTFFS KFFKQRCPDA YSYPQDDPTS TFTCPGGSTN

Arab.PR-5      YEITFCP*
Tob.PR-5maj.   YRVVFCP*
Tob.PR-5min.   YRVVFCP*
Osmotin        YRVIFCPNGQ AHPNFPLEMP GSDEVAK*
```

Figure 6. Amino Acid Sequence Alignments with Heterologous Proteins from Tobacco.

Residues identical to the Arabidopsis sequences are shaded.

(A) PR-1 from Arabidopsis (Arab. PR-1) compared to the acidic forms of PR-1 from tobacco (Tob. PR-1a, Tob. PR-1b, and Tob. PR-1c) (Payne et al., 1988b) and the basic form of PR-1 (Tob. basic) (Payne et al., 1989).

(B) PR-2 from Arabidopsis (Arab. PR-2) compared to the acidic glucanase PR-2 from tobacco (Tob. acidic) (Ward et al., 1991a), a basic glucanase (Tob. basic) (Shinshi et al., 1988), and PR-Q' (Tob. PR-Q') (Payne et al., 1990).

(C) PR-5 from Arabidopsis (Arab. PR-5) compared to the minor and major forms of PR-R from tobacco (Tob. PR-5min. and Tob. PR-5maj.) (Payne et al., 1988a) and osmotin (Singh et al., 1989).

Peptide sequences derived from the purified Arabidopsis proteins are indicated on the top row of each panel. Periods represent gaps introduced to maximize alignment.

Further experiments, such as constitutive expression of these proteins from transgenes, will be required to confirm this hypothesis. In addition to INA, SA was able to induce strongly the expression of PR-1, PR-2, and PR-5, suggesting that SA could be an endogenous signal that mediates both gene induction and immunization in Arabidopsis.

The predicted primary translation products of the three types of cDNAs had N-terminal extensions compared to the known or presumed mature protein N termini. The presence of these putative signal peptides is assumed to mediate passage of the proteins through the secretory pathway. In tobacco, the intracellular, basically charged members of the PR-1, PR-2, and PR-5 protein families have C-terminal extensions compared to their extracellular counterparts. Such C-terminal extensions have been shown to be necessary and sufficient for vacuolar localization in tobacco and barley (Bednarek and Raikhel, 1991; Neuhaus et al., 1991). The Arabidopsis protein sequences all share conserved C termini with their extracellular tobacco heterologs, consistent with their localization in the apoplastic compartment.

Isolation of a genomic clone for PR-1 from Arabidopsis has recently been reported (Metzler et al., 1991); however, this gene is less than 60% identical to the cDNA we report here. Using a probe specific for the PR-1 genomic clone, we did not detect hybridization to RNA from INA-treated Arabidopsis or to any plaques in the cDNA library (S. Uknes, unpublished data). Therefore, the previously published sequence may not be involved in the plant's defense response. The predicted signal peptide of the PR-2 protein was somewhat unusual in its structure, suggesting that it may have arisen artifactually. However, the primary structure of this region of the cDNA was identical in at least two independent clones. Moreover, the cDNA sequence reported here exactly matches the sequence of a genomic glucanase clone (BGL2) previously described by Dong et al. (1991).

In addition to the PR-1, PR-2, and PR-5 cDNAs, Arabidopsis contains genes encoding PR-3 (chitinase) (Samac et al.,

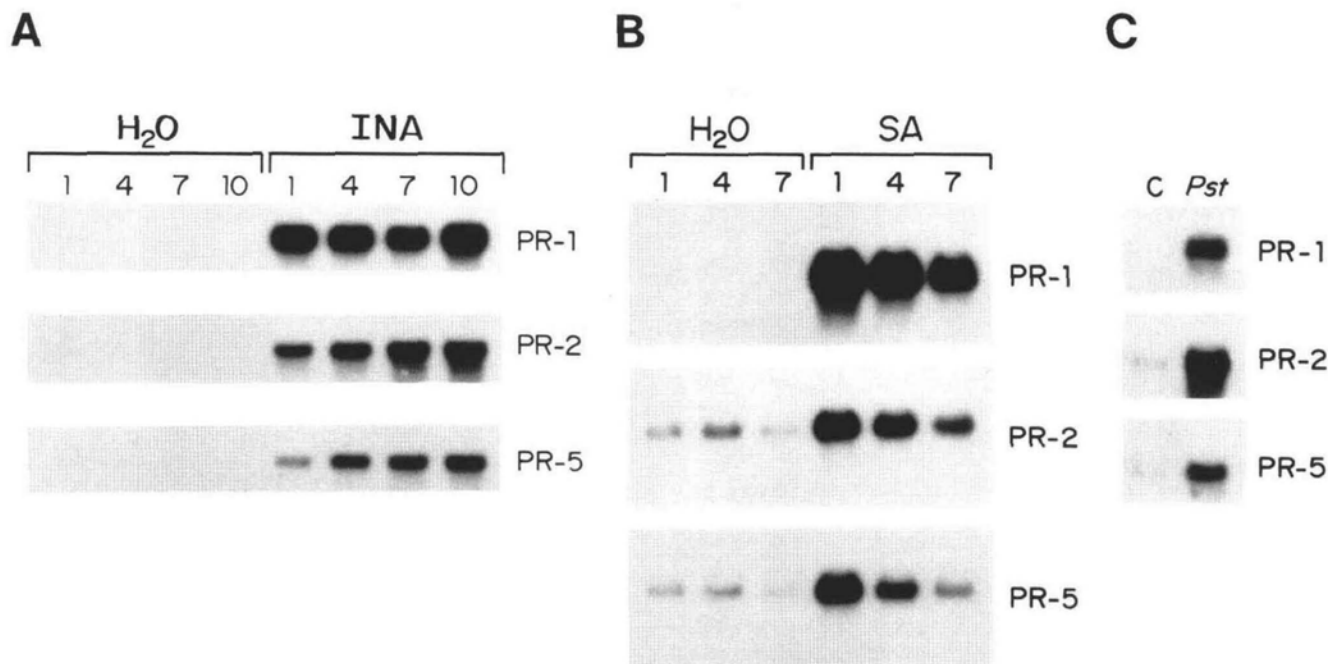

Figure 7. Gene Expression in Response to Various Stimuli.

RNA gel blot hybridizations were performed using the PR-1, PR-2, and PR-5 cDNAs as probes.
(A) INA treatment.
(B) SA treatment. Numbers above the lanes indicate days after treatment in **(A)** and **(B)**.
(C) *P.s. tomato* DC3000 infection. C, the untreated control lane; *Pst*, the lane containing RNA from tissue 6 days postinfection.

1990; Verburg and Huynh, 1991) and PR-4 (unknown function; S. Potter and S. Uknes, unpublished data). Although genes in these families are strongly inducible by both pathogens and INA in tobacco, they appear to respond only weakly to chemical treatment in Arabidopsis (S. Potter and S. Uknes, unpublished data). Other plant species, for example cucumber, express genes that are not structurally related to the abundant PR proteins of tobacco but are thought to share functional analogy (Métraux and Boller, 1986). Thus, different plants display different patterns of molecular markers in the induced resistant state. In the context of developing Arabidopsis as a model system for acquired resistance, the overall similarity of its molecular response to other species is encouraging. The work described here lays the foundation for genetic analysis of immunization in plants.

METHODS

Cultivation of Plants for Inoculation with *Peronospora parasitica*

Arabidopsis thaliana accession Weiningen and ecotype Landsberg *erecta* were sown in 200-mL containers in sterile potting compost covered with a fine layer of vermiculite. The seeds were surface-sterilized with bleach (1 to 2% active chlorine, 15 to 30 min) and then washed several times in sterile distilled water prior to sowing. For seed vernalization, the containers were kept at 4°C in the dark for several days and then transferred to a growth chamber with constant light (75 $\mu E\ m^{-2}\ sec^{-1}$) and a temperature of 20 to 23°C.

Soil Drench Treatment with 2,6-Dichloroisonicotinic Acid

INA (CIBA-GEIGY AG, Basel, Switzerland), formulated as 25% active ingredient with a wettable powder carrier (Métraux et al., 1991) or the wettable powder alone, was applied as a soil drench 1, 4, and 7 days before inoculation with *P. parasitica*. INA was applied at 0.52, 5.2, and 52 μM final concentration in the soil. The suspensions were prepared with sterile tap water.

Inoculation with *P. parasitica*

Plants were inoculated 4 weeks after sowing by spraying with a conidial suspension (approximately 10^6 spores mL^{-1}). Inoculated plants were incubated overnight in a moist chamber at 20°C and then returned to the growth chamber. Seven (Weiningen) and 10 days (Landsberg *erecta*) after inoculation, the plants were again incubated overnight in a moist chamber to induce sporulation. Plants were scored for conidiophores using a magnifying glass (4×), and every day after

inoculation 10 leaves were harvested randomly and stained with lactophenol–trypan blue (Keogh et al., 1980) for examination under the microscope.

Plant Material and *Pseudomonas syringae* pv *tomato* Infection Conditions

Arabidopsis ecotype Columbia (Lehle Seeds, Tucson, AZ) was sown in an all-purpose soil mix that had been autoclaved two times for 70 min. The plants were placed in growth chambers at 22°C, 50% relative humidity, 9-hr day/15-hr night. The soil surface was allowed to dry between watering. When the plants were approximately 4 weeks old, they were sprayed with either water or INA (0.65 mM). (INA is effective in root drench application at a concentration 10- to 100-fold lower than that required for equivalent efficacy by foliar spray [Métraux et al., 1991].) Nine days later, *P.s. tomato* infections were carried out by dipping plants in a suspension of bacteria (10^8 colony-forming units per mL) in 10 mM $MgCl_2$, 0.01% Silwet L-77 (Union Carbide, Danbury, CT), as described by Whalen et al. (1991). For determining the growth of *P.s. tomato* in Arabidopsis, plants were sprayed with INA (0.65 mM) or wettable powder. Nine days later, the plants were dipped in a suspension of *P.s. tomato* DC3000 as described above. One and 4 days after infection, bacteria were extracted with 10 mM $MgCl_2$ from 1 g of leaves, and appropriate dilutions were plated on nutrient agar (Difco) containing 50 µg of rifampicin per milliliter.

Protein Chemistry

PR-1 Protein

Intercellular fluid (ICF) from Arabidopsis plants treated with INA was electrophoresed on a 10 to 20% Novex mini gel (San Diego, CA) under denaturing conditions in Tris-tricine buffer. A 16-kD protein was excised along with a lower molecular weight contaminant and electroeluted using an Elutrap (Schleicher & Schuell, Keene, NH). Purification was achieved using a phenyl column (Polypore; Brownlee Labs, San Jose, CA) with a linear gradient of 0 to 50% acetonitrile/isopropanol (1:1) in 0.1% trifluoroacetic acid. The amino terminus was blocked to Edman degradation, so the protein (unmodified) was digested with Lys-C and trypsin (Boehringer Mannheim, Indianapolis, IN) in 0.1 M Tris-HCl, pH 8.5, for 48 hr at 37°C with an enzyme-to-substrate ratio of 1:50. The protein was reduced and alkylated with 4-vinylpyridine by incubating samples in 6 M guanidine-HCl, 1 M Tris-HCl, pH 8.6, 10 mM EDTA, and 20 mM DTT for 1 hr at 37°C. 4-Vinylpyridine was added to 50 mM, and incubation was continued for 1 hr at room temperature. The modified protein was desalted on a phenyl column (Polypore; Brownlee Labs) with a linear gradient of 0 to 80% acetonitrile/isopropanol (1:1) in 0.1% trifluoroacetic acid. The protein was digested with endoproteinase Lys-C (Wako) in 0.1 M Tris-HCl, pH 8.5, for 18 hr with an enzyme-to-substrate ratio of 1:50. Peptides were separated on a reverse phase column (model No. RP 300; Brownlee Labs) with a linear gradient of 0 to 60% acetonitrile/isopropanol (1:1) in 0.1% trifluoroacetic acid. Automated Edman degradations were performed using a sequencer (model No. 475A; Applied Biosystems, Foster City, CA). Phenylthiohydantoin amino acids were identified with an on-line phenylthiohydantoin analyzer (model No. 120A; Applied Biosystems).

PR-2 Protein

Intercellular fluid from Arabidopsis plants treated with INA was electrophoresed under nondenaturing conditions using a 17 to 27% miniSepragel (Integrated Separation Systems, Hyde Park, MA) in Tris-glycine buffer, pH 8.3. The protein band was electroblotted onto ProBlott (Applied Biosystems) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0, containing 10% methanol for 15 min at 50 V, stained with Coomassie Brilliant Blue R250, excised, and electroeluted. After electroelution, the protein was desalted on a phenyl column (Polypore; Brownlee Labs), and peptides were isolated and sequenced as was done for PR-1.

PR-5 Protein

The protein was electroblotted from native polyacrylamide gels as described for PR-2. An N-terminal sequence was obtained for 25 residues with no residue identified at cycle 9. The cysteine residues were not modified because the protein was sequenced from the blot. Protein for proteolytic digestion was obtained by electrolution from a nondenaturing gel using an Elutrap (Schleicher & Schuell) in Tris-glycine, pH 8.3. Peptides were separated and sequenced as described for PR-1.

cDNA Cloning

A λ ZapII (Stratagene, La Jolla, CA) cDNA library was made from poly(A)$^+$ RNA from leaves of 4-week-old chemically treated Arabidopsis plants. The Arabidopsis PR-1 cDNA was isolated by screening the cDNA library at low stringency with a mixture of both the PR-1a (Payne et al., 1988b) and PR-1basic (Payne et al., 1989) cDNAs from tobacco as probes. The cDNA library was plated at 5000 plaque-forming units per plate, and filter lifts were taken with nitrocellulose filters (Ausubel et al., 1987). Probes were made by the random-priming method (Feinberg and Vogelstein, 1983) with a Prime Time C kit (International Biotechnologies, Inc., New Haven, CT). Hybridization and washing were essentially as described by Church and Gilbert (1984) except that both were performed at 50°C. The PR-2 cDNA from Arabidopsis was isolated by first designing a degenerate oligonucleotide that corresponded to a peptide sequence (MFDENK) derived from the purified protein (Figure 6B). This oligonucleotide (5′-ATGTTYGAYGARAAYAA-3′) was then used to screen the cDNA library under low-stringency conditions as described for PR-1. For PR-5, two fully degenerate oligonucleotides were designed that correspond to the peptide sequences NNCPTT and DQYCCR. These oligonucleotides were used with a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) to prime a polymerase chain reaction on double-stranded cDNA template. The resulting 500-bp band was isolated from a low-melting-point agarose gel and used to screen the cDNA library under standard hybridization conditions (Church and Gilbert, 1984). After isolation of single independent hybridizing plaques, in vivo excision was performed and plasmid DNA was isolated (Qiagen, Chatsworth, CA) and sequenced (Hattoni and Sakaki, 1986). Sequence comparisons were performed using the GAP and PILEUP programs (Deveraux et al., 1984).

Analysis of Gene Expression

RNA was purified from frozen tissue samples by phenol/chloroform extraction followed by lithium chloride precipitation (Lagrimini et al.,

1987). Samples of total RNA (5 μg) were separated by electrophoresis through formaldehyde-agarose gels and blotted onto nylon membrane (GeneScreen Plus; NEN Research Products, Boston, MA) as described by Ausubel et al. (1987). Ethidium bromide was included in the sample loading buffer at a concentration of 40 μg/mL, which allowed photography under UV light after electrophoresis to confirm equal sample loading. Hybridizations and washing were according to Church and Gilbert (1984). Relative amounts of transcript were determined by detecting β-decay of phosphorous-32 with a blot analyzer (Betascope 603; Betagen, Waltham, MA).

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