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Analysis of the Synthesis of Several Pathogenesis-Related Proteins in Tobacco Leaves Infiltrated with Water and with Compatible and Incompatible Isolates of *Pseudomonas solanacearum*

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Pathogen attacks and treatment of many plant species by various chemicals induce the synthesis of pathogenesis-related (PR) proteins. These host-encoded, low molecular mass proteins have been well studied in tobacco reacting hypersensitively to infection by TMV. A partial cDNA clone encoding for β-1,3-glucanase was used as a probe to study the kinetics of accumulation of the corresponding mRNAs in tobacco leaves infiltrated with water or with compatible (K60), incompatible (GMI1000), and avirulent (GMI1178) isolates of a phytopathogenic bacterium, *Pseudomonas solanacearum*. A nonspecific accumulation of these transcripts, independent of the nature of the inoculum, was observed. Similar results were obtained with pCHN50, a chitinase-encoding cDNA clone. In addition, antibodies directed against several PR proteins were used to estimate the accumulation of these proteins in tobacco leaves infiltrated for 18 hr with the same *P. solanacearum* isolates or with water: no qualitative or quantitative difference could be detected. These data show that the stress provoked by our technique of infiltration of bacteria in leaves induces a nonspecific accumulation of these PR proteins. In addition, our results indicate that these polypeptides are not sufficient to provoke the appearance of necrotic lesions associated with the hypersensitive reaction.

Additional keywords: mRNA accumulation, *Nicotiana tabacum*.

In response to infection by an incompatible (nonhost or avirulent) pathogen, many plant species have been shown to develop a hypersensitive reaction (HR). This response is characterized by the rapid necrosis of plant cells at the site of inoculation, which restricts the spread of the pathogen and eventually leads to its death (Klement 1982; Goodman *et al.* 1986).

Pathogenesis-related (PR) proteins were first identified in tobacco cells reacting hypersensitively to tobacco mosaic virus (TMV) (Gianinazzi *et al.* 1970; Van Loon and Van Kammen 1970) and were subsequently described in a number of plant species after infection by various pathogens (Redolfi 1983) and treatment with specific chemicals (Van Loon 1983). The activities of some of these proteins have been recently determined. Several PR proteins have hydrolytic activities, for example β-1,3-glucanase (Kauffmann *et al.* 1987; Kombrink *et al.* 1988) and chitinase (Legrand *et al.* 1988). Another PR protein, PR-S, shares extensive homology with the sweet-tasting thaumatin and to a wheat α-amylase protease inhibitor (Cornelissen *et al.* 1986; Richardson *et al.* 1987).

Our goal is to study the molecular events leading to the HR after inoculation of tobacco with an incompatible isolate of a bacterial pathogen, *Pseudomonas solanacearum* (Smith) Smith (Buddenhagen and Kelman 1964). This bacterium, the causative agent of bacterial wilt, was chosen because of our knowledge of its genetics and the existence of various isolates and mutants able to induce different plant responses. Indeed, a typical race 1 isolate, K60, provokes the disease, whereas an atypical race 1 isolate, GMI1000, induces the development of an HR within 24 hr after infiltration. A derivative of the latter strain, called GMI1178, was shown to be deleted for some *hrp* genes involved in the HR and has no apparent effect on the inoculated leaves (Boucher *et al.* 1986, 1988; Message *et al.* 1978). Preliminary studies on the mRNA activities from tobacco leaves infiltrated with the aforementioned *P. solanacearum* isolates indicate that important changes in the translation products are detectable within 6 hr after inoculation with the GMI1000 isolate. At this time, both the K60 and GMI1178 isolates have no detectable effect (Ragueh *et al.* 1989).

Using this system, the possible involvement of PR proteins in the formation of necrotic lesions characteristic of the HR was investigated. Therefore, the kinetics of accumulation of transcripts corresponding to cDNA clones encoding for β-1,3-glucanase and chitinase were determined in tobacco leaves infiltrated with the different *P. solanacearum* isolates. In addition, the accumulation of several PR proteins was estimated in the late stages of the HR and in the other interactions using polyclonal antibodies directed against PR-O, PR-R, PR-Q, and PR-1b. These experiments show that the accumulation of mRNAs and proteins corresponding to the PR proteins studied is provoked by the inoculation procedure. Moreover, the observation that several PR proteins accumulate in leaves not undergoing necrosis as well as in leaves undergoing necrosis

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suggests that these polypeptides are not sufficient to induce the formation of necrotic lesions associated with the HR.

MATERIALS AND METHODS

Plant material and conditions of inoculation. Seedlings of *Nicotiana tabacum* L. cv. Bottom Special (gift from Luis Sequeira, University of Wisconsin, Madison) were grown in a greenhouse for 6 wk and then transferred to a growth chamber where they were maintained for 3 wk. Leaves were then detached and infiltrated *in vacuo* with the bacterial suspension as previously described (Ragueh *et al.* 1989).

Bacterial strains. All the *P. solanacearum* strains used in this study (GMI1000, GMI1178, and K60) were kindly provided by C. Boucher (CNRS-INRA, Toulouse, France). All these strains were grown at 30° C in BGT medium (Boucher *et al.* 1985) and conserved at 4° C in one-quarter strength M_{63} (Maniatis *et al.* 1982).

The GMI1178 strain is a methionine auxotroph, and the medium was supplemented with L-methionine (16 mg/L).

Nucleic acid isolation. For each RNA extraction, at least three leaves from different tobacco plants were used. The RNA was prepared according to the method described previously (Ragueh *et al.* 1989). Poly(A)$^+$ RNA was purified by two passages on oligo(dT)-cellulose columns (Rochaix and Malnoe 1982).

Plasmid DNA was extracted by the alkaline lysis method (Maniatis *et al.* 1982). After digestion with the appropriate restriction enzyme, the inserts were separated from the vector (pBR322) on agarose gels, excised, and then purified by electroelution (Chouikh *et al.* 1979).

Labeling of the DNA inserts. The inserts were labeled by the random primer technique (Feinberg and Vogelstein 1983, 1984).

RNA transfers and hybridization conditions. Total RNA was electrophoresed on 1.5% agarose gels containing methyl mercury (Maniatis *et al.* 1982). Each sample was run on two parallel gels. One was stained with ethidium bromide, and the other transferred on a Genescreen membrane according to the recommendations of the manufacturer (New England Nuclear, Boston).

Total RNA was spotted on the same membrane using a Hybri-Dot apparatus (Bethesda Research Laboratories, Gaithersburg, MD).

Hybridizations and washings of the Genescreen filters were done essentially as suggested by the manufacturers.

cDNA clones used in this study. cDNA clone pNt239 (1,000 base pairs [bp]) was isolated from a cDNA library prepared with mRNAs isolated from tobacco leaves infiltrated with the GMI1000 strain 6 hr after infiltration. A differential screening of this cDNA library was performed using ^{32}P-labeled cDNAs synthesized from mRNAs isolated from leaves infiltrated for 6 hr with the GMI1000 strain or with the GMI1178 strain. Several "HR-associated" cDNA clones, such as pNt239, were isolated by this procedure (Y. Marco *et al.*, unpublished data).

cDNA clone pNt517 (400 bp) was isolated from the same library using a homologous tobacco β-1,3-glucanase-encoding cDNA clone (Shinshi *et al.* 1988). Its nucleotide sequence was determined and is identical to the sequence of cDNA clone pGL43 (Shinshi *et al.* 1988). The inserts were obtained by *Pst*I digestion of the plasmid DNAs.

The human ubiquitin-encoding cDNA clone (pHUb14-38) was a kind gift of O. Wiborg (University of Aarhus, Denmark). Its DNA insert was excised by *Xho*I digestion of the plasmid DNA (Wiborg *et al.* 1985).

The chitinase-encoding cDNA clone (pCHN50) was obtained from F. Meins, Jr. (Friedrich Miescher Institute, Basel, Switzerland).

Preparation of protein extracts, analytical two-dimensional electrophoresis, staining, and immunodetection. Total proteins were purified by phenol extraction and separated by two-dimensional gel electrophoresis. Proteins were transferred onto nitrocellulose sheets as described by Meyer *et al.* (1988).

Each sample was run on two parallel gels: one was electrotransferred and the other stained with Coomassie Brilliant Blue R 250.

Immunodetection of PR proteins was performed as previously described (Grosset *et al.* 1990) with polyclonal antibodies (a gift of B. Fritig, Strasbourg, France) raised against four PR proteins extracted from *N. tabacum* cv. Samsun NN infected by TMV (Jamet and Fritig 1986; Legrand *et al.* 1988; Kauffmann *et al.* 1987; Linthorst *et al.* 1989).

RESULTS

Kinetics of steady-state levels of mRNAs corresponding to a β-1,3-glucanase-encoding cDNA clone (pNt517) and a chitinase-encoding cDNA clone (pCHN50) during interactions of tobacco leaves with various isolates of *P. solanacearum*. For this experiment, total RNA from leaves infiltrated with the compatible (K60), the incompatible (GMI1000), the deleted strain (GMI1178), or with water was purified after various periods of incubation. After electrophoresis and transfer onto a membrane, these RNAs were probed with the purified ^{32}P-labeled 400-bp insert of pNt517 (discussed previously in the text). The results (Fig. 1) indicate that a 1.7-kilobase (kb) transcript corresponding to β-1,3-glucanase mRNAs accumulates whatever the nature of the inoculum. This transcript appeared between 2 to 4 hr after infiltration, and its level increased until 36 hr. A smear, probably due to the partial degradation of these mRNAs, was detectable in some samples. Several discrete bands were visible, suggesting that the 1.7-kb transcript is cleaved at specific sites during this process.

A control consisting of similar amounts of total RNA purified from leaves inoculated with the GMI1000 isolate and incubated for 6 hr allowed us to compare the accumulation of β-1,3-glucanase mRNAs in the various experiments (Fig. 1, lanes Con. of panels A, B, C, D, and E). Moreover, the rRNA levels of the different samples were compared after ethidium bromide staining of the different gels and found to be comparable (Fig. 1, panel A).

The method of inoculation, which involves leaf detachment and infiltration of the bacterial suspension or water *in vacuo*, induced a mechanical stress responsible for the observed increase of the 1.7-kb transcript: indeed, the corresponding mRNAs accumulated in control leaves infiltrated with water. In the incompatible interaction, this

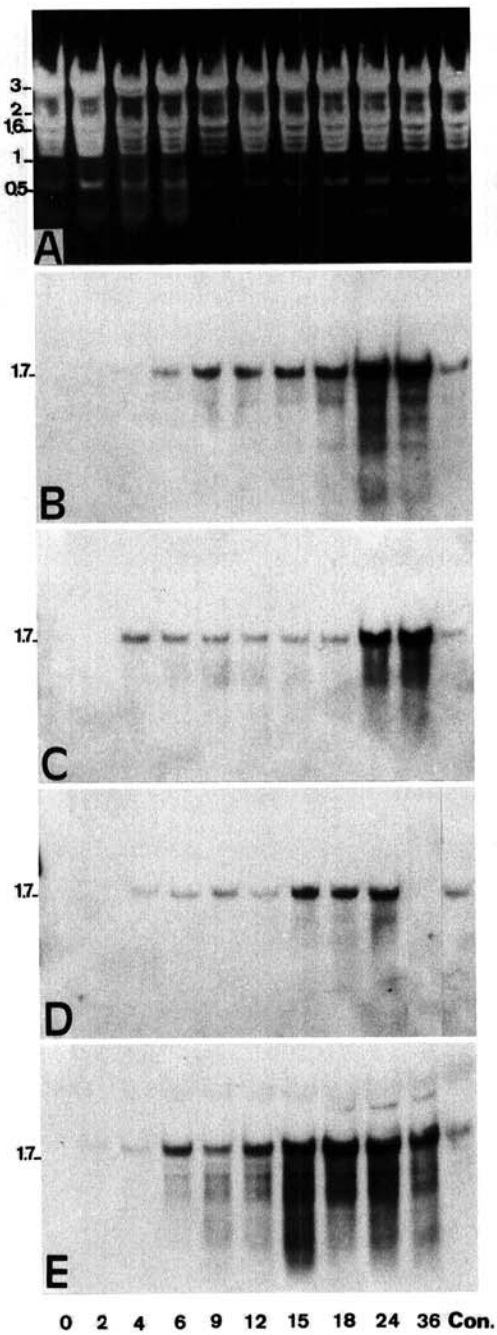

Fig. 1. Ten micrograms of total RNA prepared from leaves infiltrated with water (**B**) or the GMI1178 (**C**), the GMI1000 (**D**), or the K60 (**E**) isolate was electrophoresed on a 1.5% agarose gel containing methyl mercury. Moreover, 10 μg of total RNA isolated from leaves infiltrated with the GMI1000 isolate was loaded on each gel (lanes Con. of **A**, **B**, **C**, **D**, and **E**). These RNAs were transferred onto a membrane and hybridized to the ^{32}P-labeled insert of the β-1,3-glucanase-encoding cDNA clone (pNt517). The autoradiograms were exposed for 4 days at −80° C with intensifying screens. In **A**, the ethidium bromide staining of a duplicate gel is shown. RNA size was estimated by interpolation from the migration of standard marker DNAs (Boehringer, Mannheim, Federal Republic of Germany, 1-kilobase DNA ladder). Hours after infiltration and lanes Con. (lanes on the far right-hand side) are indicated under the figure.

transcript was detected within 4 hr after inoculation of the GMI1000 strain. No early transient increase in the amount of this mRNA was observed in the first hours of the HR. In addition, in repeated experiments, the steady-state levels of this transcript found until 12 hr in the incompatible interaction were lower or comparable to those detected in leaves infiltrated with water or with the GMI1178 and K60 strains.

Similar experiments were performed with pCHN50, a basic chitinase-encoding cDNA clone that hybridizes with a single 1.3-kb RNA species (Shinshi *et al.* 1987). The results (Fig. 2) indicate that the accumulation of this transcript does not appear to depend on the presence or nature of the bacteria. The mRNAs hybridizing to this probe appeared as soon as 2 hr and continued to increase until 24 hr after leaf infiltration with water or bacteria (Fig. 2, panel I). The same blot was hybridized with an ubiquitin-

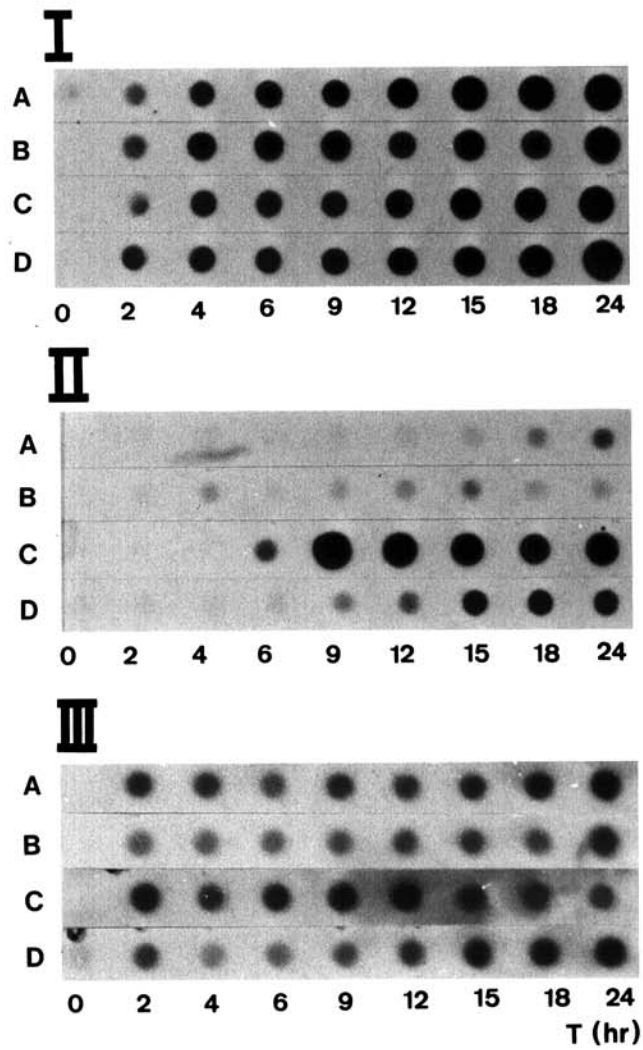

Fig. 2. Ten micrograms of total RNA from leaves infiltrated with water (A) or the GMI1178 (B), the GMI1000 (C), or the K60 (D) isolate was spotted onto Genescreen membranes using a Bethesda Research Laboratories Hybri-Dot apparatus and hybridized to the ^{32}P-labeled insert of pCHN50, the chitinase-encoding cDNA clone (panel **I**); cDNA clone pNt239 (panel **II**); and an ubiquitin-encoding cDNA clone (panel **III**). The autoradiograms were exposed for 72 hr at −80° C. Hours after infiltration are indicated under each panel.

encoding cDNA clone to check that similar amounts of RNA were spotted on the filters (Fig. 2, panel III).

These two results could be explained by a lack of specificity of our system. Therefore, the kinetics of accumulation of a cDNA clone, such as pNt239 (described previously in the text), whose transcript accumulates in leaves undergoing an HR were determined using the same biological material (Fig. 2, panel II). With this probe, hybridizable RNAs accumulated to a high level 6 hr after inoculation with the incompatible strain and later, after 9 to 12 hr, to a lesser extent in the compatible interaction. In the control (water-treated leaves), the levels of transcripts remained quite low. This result eliminates the possibility that the harsh conditions of infiltration of the inocula suppress all specificity of the plant responses in our system.

Synthesis of several PR proteins in tobacco leaves infiltrated with different *P. solanacearum* isolates or water. To test whether the HR leads to a specific increase in the amount of some PR proteins, the total accumulation of several of these proteins was determined in leaves 18 hr after infiltration with strains K60, GMI1000, and GMI1178 or with water. At this time, the first symptoms of necrosis were already visible in leaves infiltrated with the incompatible isolate. We have assumed that proteins detected then result from the sum of proteins present in uninfiltrated leaves and proteins synthesized during the 18 hr after inoculation: indeed, it is known that PR proteins are both very stable and highly resistant to proteolytic degradation. Therefore, any detectable difference in the amount of a given PR protein after 18 hr of incubation

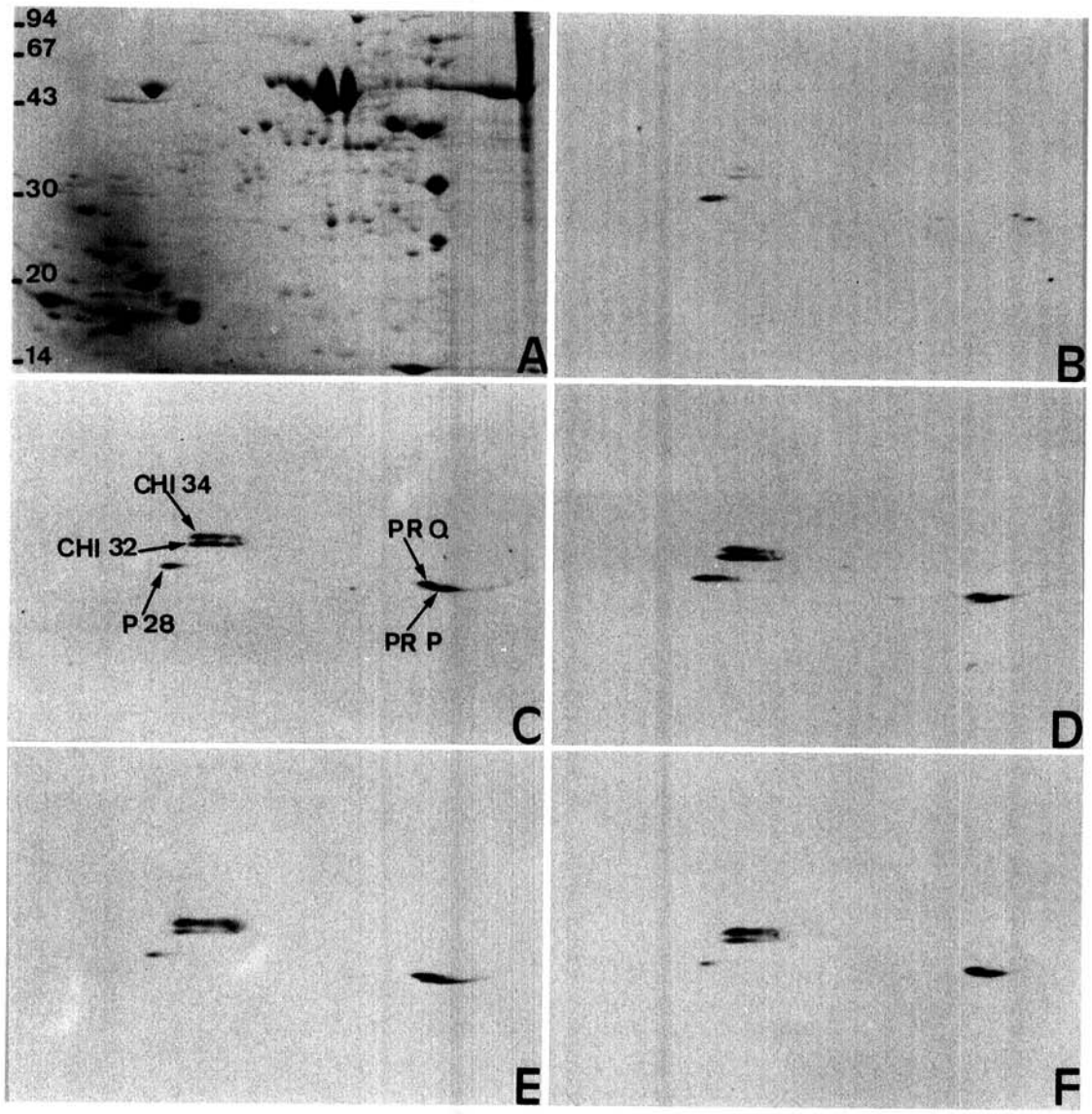

Fig. 3. Detection of chitinases in protein extracts from leaves infiltrated with water, **B** (at time 0); the GMI1178 isolate, **C** (at 18 hr); water, **D** (at 18 hr); the GMI1000 isolate, **E** (at 18 hr); and the K60 isolate, **F** (at 18 hr). Polypeptides were separated by two-dimensional gel electrophoresis and electrotransferred onto nitrocellulose. In **A** is an example of Coomassie blue staining of the two-dimensional gels before electroblotting is shown. The molecular mass markers (in kDa) are indicated on the left side of **A**. Pathogenesis-related (PR) proteins were then immunodetected with antibodies raised against PR-Q. The positions of the two acidic chitinases, PR-P and PR-Q, the basic chitinases (molecular masses, 32 and 34 kDa), and of an unidentified protein of 28 kDa (P28) are indicated with arrows in **C**. For all samples, the pH gradient is from left (basic) to right (acidic).

should reflect a differential synthesis of this protein during a particular plant-pathogen interaction. We used four different antibodies that permitted the detection of all currently described tobacco PR proteins.

The anti-PR-Q antiserum reacts with PR-Q and PR-P proteins, the two acidic chitinases (molecular mass of about 28 kDa) and also with the two basic chitinases CHI-32 (molecular mass of about 32 kDa) and CHI-34 (molecular mass of about 34 kDa) (Legrand *et al.* 1988), as well as with a basic polypeptide of 28 kDa whose function is still unknown (Grosset *et al.* 1990). Small amounts of these five polypeptides were detectable in the uninfiltrated leaf (Fig. 3, panel B). Eighteen hours after infiltration, the levels of both acidic and basic chitinases clearly increased (Fig. 3, panels C, D, E, and F). The accumulation of these polypeptides does not appear to depend on the presence or nature of the bacteria.

The anti-PR-O antiserum reacts with acidic β-1,3-glucanases PR-O, PR-N, and PR-2 (molecular mass of about 40 kDa) as well as with the basic β-1,3-glucanase (molecular mass of about 32 kDa) (Kauffmann *et al.* 1987). Very small amounts of the basic β-1,3-glucanase were present in the water-treated leaf at time 0 (Fig. 4B, panel 2). Eighteen hours after infiltration, the amount of this polypeptide increased independently of the presence and nature of the bacteria (Fig. 4B, panels 1, 3, 4, and 5). No acidic β-1,3-glucanase was detected during the course of these experiments.

The anti-PR-S antiserum reacts with the acidic polypeptide (molecular mass of about 26 kDa) PR-S that shows extensive sequence homology with a maize protein inhibiting the activity of proteinase and α-amylase (Linthorst *et al.* 1989). Tobacco leaves contain low levels of PR-S (Fig. 4C, panel 2). Eighteen hours after water infiltration, an increase in the level of this polypeptide was observed, its level being independent of the presence or nature of the bacteria (Fig. 4C, panels 1, 3, 4, and 5).

The anti-PR-1b antiserum reacts with the acidic polypeptides PR-1a, PR-1b, and PR-1c (molecular mass of about 14.5 kDa) (Grosset *et al.* 1990). No PR-1 was detected in our tobacco leaves either before or 18 hr after infiltration with water or bacteria (data not shown).

DISCUSSION

In this study, the possible role played by PR proteins in the formation of necrotic lesions, characteristic of the HR, was investigated. Indeed, these proteins have been associated with this particular plant response in a number of plants challenged by various pathogens (Redolfi 1983). The method of inoculation used in this study allowed us to simultaneously infiltrate the whole leaf with the bacteria. Therefore, one can reasonably assume that the molecular events which took place in the infected cells were well-synchronized. However, this technique, which involves leaf detachment followed by infiltration of bacteria *in vacuo*, is quite stressful. Our results confirmed this stressful effect: it induced an accumulation of basic as well as acidic chitinases, basic β-1,3-glucanase, and PR-S after 18 hr. At this time, acidic glucanases and PR-1 were not detectable. However, at a late stage after water infiltration (48 hr), they became detectable (data not shown).

Nevertheless, it should be noted that at 18 hr after infiltration, the level of PR proteins was extremely low,

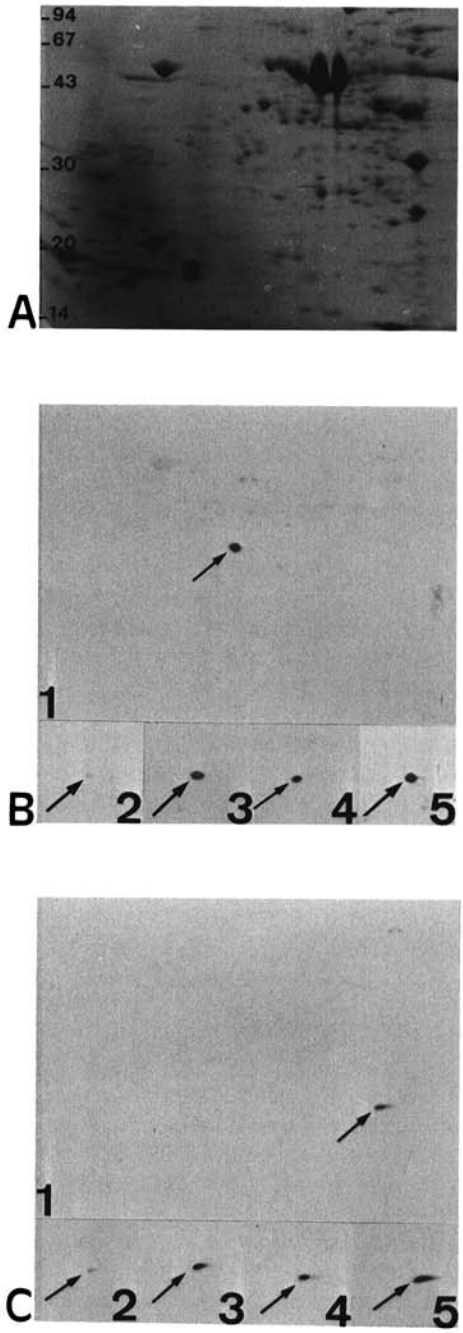

Fig. 4. Detection of several pathogenesis-related (PR) proteins in extracts from leaves infiltrated with the GMI1000 isolate, panel 1 (at 18 hr); water, panel 2 (at time 0); water, panel 3 (at 18 hr); the GMI1178 isolate, panel 4 (at 18 hr); and the K60 isolate, panel 5 (at 18 hr). The conditions of the experiment are the same as in Figure 3. In **A** is an example of Coomassie blue staining of the two-dimensional gels before electroblotting is shown. The molecular mass markers (in kDa) are indicated on the left side of **A**. The antibodies used in this experiment were raised against PR-O (**B**) and against PR-S (**C**). In **B**, the arrow shows the position of the basic β-1,3-glucanase (molecular mass of about 32 kDa) and in **C**, the position of PR-S (molecular mass of about 26 kDa).

in comparison with Samsun NN tobacco leaves, 7 days after TMV infection. In fact, PR proteins were not detectable in Coomassie blue-stained two-dimensional gel patterns (data not shown), and on immunoblots we obtained comparable signals by using 50 to 100 times less total protein extracted from virus-infected leaves. Thus, although clearly established, PR protein accumulation in tobacco leaves 18 hr after infiltration remained very low.

In addition, our results show that the accumulation of PR proteins is quantitatively and qualitatively similar whatever the nature of the inoculum, that is the same in tobacco leaves infiltrated with GMI1000 undergoing necrosis as in tobacco leaves not undergoing necrosis. This strongly suggests that the accumulation of these PR proteins is not sufficient to induce the development of lesions associated with the HR. Nevertheless, it cannot be excluded that PR protein synthesis and accumulation start earlier in leaves undergoing necrosis. This is unlikely: in the first hours of the incompatible interaction, the kinetics of accumulation of the mRNAs of a basic β-1,3-glucanase indicated in repeated experiments that the amount of this 1.7-kb transcript was lower or comparable to that found in leaves not undergoing necrosis. In addition, a similar experiment was performed with a basic chitinase-encoding cDNA clone. The data indicated that the corresponding mRNAs accumulated whatever the nature of the inoculum and that no early difference in the amount of the chitinase transcript was detected in the HR versus the other plant responses. The observation that during the different interactions, the same polypeptides cross-react with the anti-PR-Q antiserum makes it unlikely that different chitinase genes might be expressed in leaves both undergoing and not undergoing necrosis.

In our system, no PR-1 protein could be detected in leaves undergoing necrosis. These data are consistent with previous reports (Antoniw and White 1983; Carr *et al.* 1987) showing that PR-1 proteins are localized predominantly in regions adjacent to viral lesions and present in low amounts in the center of the lesion. They suggested that plant cells in the central zone become necrotic before they are able to synthesize large amounts of PR proteins. These observations indicate that this particular class of PR proteins, if required for the formation of necrotic lesions, must be active in very low amounts. In fact, the same conclusion applies to acidic glucanases that are not detectable either in leaves undergoing or not undergoing necrosis 18 hr after infiltration. Moreover, the levels of basic glucanase, basic chitinases, acidic chitinases, and PR-S do not differ significantly in leaves undergoing or not undergoing necrosis.

Our results complement the recent report of Linthorst *et al.* (1989). They constructed transgenic tobacco plants that constitutively synthesized PR-1 and PR-S proteins. These plants presented a normal morphology without necrotic symptoms. Thus, it appears that constitutive synthesis of individual PR proteins does not result in necrosis and, furthermore, that necrosis is induced under conditions where some PR proteins are not detectable and others are accumulated only at a very low level. Therefore, it appears that the synthesis of several PR proteins is not sufficient to explain the development of lesions although they may play a major role during the HR in the areas surrounding these necroses.

"HR-associated" or "necrosis-associated" cDNA clones (such as pNt239) have been isolated. The kinetics of accumulation of the corresponding mRNAs during the different plant responses (Y. Marco *et al.*, unpublished data) indicate that the products of genes encoding these transcripts might be tightly linked to the formation of necrotic lesions. Such genes might encode for proteins associated with some physiological changes taking place during the HR or for enzymes implicated in the production of toxic compounds that could provoke the death of the bacteria and/or host cell. These cDNA clones are now being characterized.

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