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Defense Reaction in *Medicago sativa*: A Gene Encoding a Class 10 PR Protein Is Expressed in Vascular Bundles

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Infiltration of *Medicago sativa* leaves with a suspension of *Pseudomonas syringae* pv. *pisi* elicits the accumulation of several mRNA classes. A clone, designated as *Ms*PR10-1, encoding a polypeptide exhibiting strong similarity to the class 10 PR protein was isolated and characterized from a cDNA library prepared from leaf mRNA. The corresponding gene was shown to be developmentally regulated: Except in roots, its expression was not detectable in other analyzed organs of healthy plants (hypocotyls, cotyledons, stems, leaves, and flower buds). *Ms*PR10-1 transcript accumulation was especially high in leaf blades during an incompatible interaction: It was already detectable 3 h after infection, reached its maximum level 24 h postinfection, and remained at a high level over a period of at least 72 h. In addition, the expression of this gene was induced by salicylic acid treatment of the leaves. Southern hybridizations showed that this gene belongs to a multigene family. Using a 5′ extension technique for cDNA, we demonstrated that during the incompatible interaction with *P. syringae* pv. *pisi* several genes or allelic variants of this class were expressed. Measurements of transcript accumulation in both the infiltrated and noninfiltrated zones by Northern and in situ hybridizations allowed to demonstrate the "systemic" expression pattern of the *Ms*PR10-1. In situ hybridizations indicated that *Ms*PR10-1 was expressed in the vascular bundles adjacent to and distant from the infection site.

Additional keywords: incompatible interaction; infiltrated/noninfiltrated tissues; organs; PR10 expression.

Infection of plants with pathogens results in the induction of various biochemical responses. Accumulation of antimicrobial phytoalexins, enhancement of activity of certain hydrolytic enzymes and de novo synthesis of proteins termed "pathogenesis-related" (PR) proteins are among the components of the defense mechanisms induced during an incompatible interaction (reviewed, for example, by Lamb et al. 1989 or Keen 1992).

PR proteins were first identified in tobacco leaves exhibiting a hypersensitive reaction to tobacco mosaic virus (TMV) (reviewed by Bol et al. 1990), and were described for a number of plant species infected by various pathogens. Elucidation of the physico-chemical properties of these proteins as well as their biological functions has led to their classification into five classes in tobacco (reviewed by Stintzi et al. 1993). Recently, van Loon et al. (1994) proposed to extend the initial grouping to 11 families (PR-1 to PR-11) where the term "pathogenesis-related proteins" corresponds to "plant proteins that are induced in pathological and related situations."

We have developed a program aimed at analyzing plant genes expressed during an incompatible interaction between *Medicago sativa* and *Pseudomonas syringae* pv. *pisi*. Our previous reports (Esnault et al. 1993; Sallaud et al. 1995) dealt mainly with genes (CHS, CHR, CHI, and IFR) involved in the flavonoid branch pathway together with preliminary data about the involvement of a PR protein in this interaction. A PCR amplified DNA was obtained (Esnault et al. 1993) by using primers deduced from the comparison of PR sequences from parsley (Somssich et al. 1988), pea (Fristenky et al. 1988), and soybean (Crowell et al. 1992). The latter proteins, with a size equivalent to members of the tobacco PR-1 class (Somssich et al. 1986), were shown to be structurally unrelated to this class (Somssich et al. 1988). More recently, homologous sequences were identified in potato (Matton and Brisson, 1989), white birch (Breiteneder et al. 1989), and bean (Walter et al. 1990). All these PR proteins, sometimes designated as IPR (intracellular pathogenesis-related) proteins (Walter et al. 1990) and characterized in the data banks by the BetvI family signature, i.e., a conserved region located in the third quarter of the molecule, constitute now the PR-10 class (Van Loon et al. 1994).

In this communication, we report on a full-length cDNA, encoding a class 10 PR protein and we show that it belongs to a multigene family. By using a 5′ extension PCR technique (Breda et al. 1996), we demonstrated that several members of the family or allelic variants were expressed in *M. sativa* leaves during an incompatible interaction with *P. syringae* pv. *pisi*. Northern hybridizations showed that the transcripts were

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accumulated both in the injection site and the noninfiltrated zone and that the expression of this gene was also induced by salicylic acid treatment of the leaves. By in situ hybridization the strongest expression of this gene was detected in the vascular parenchyma cells, supporting its involvement in defense reaction, both in the infection site and in more distant cells.

RESULTS

Transcript characterization.

We have previously isolated a PCR-amplified fragment from a cDNA population synthesized from polyadenylated RNA extracted 6 h after inoculation of *M. sativa* leaves with a suspension of *P. syringae* pv. *pisi,* leading to an incompatible interaction (Esnault et al. 1993). This DNA fragment (320 bp) was used for screening a λgt10 cDNA library constructed from the same cDNA population. Several clones were isolated; based on the insert sizes and sequencing, none of them corresponded to full-length cDNAs (data not shown). To obtain a full length cDNA we applied a PCR 5′ extension primer technique (Breda et al. 1996). Briefly, PCR amplification was carried out by using oligonucleotide pairs designed from a region of the PR protein of interest and from the adaptor ligated to the cDNAs prior to their integration to the phage λ DNA used for constructing a cDNA library. Application of this technique for the longest cDNAs cloned (clone C7, truncated at around 60 nucleotides downstream of the putative ATG start codon), allowed us to get a full-length sequence (data not shown). The calculated molecular mass of the deduced polypeptide consisting of 157 amino acid residues (16,625 Da) is in good agreement with the estimated size (Esnault et al. 1993) of the transcript (0.8 kb). The theoretical pI of the encoded polypeptide is 4.3; moreover, no signal peptide was detected suggesting its cytosolic location.

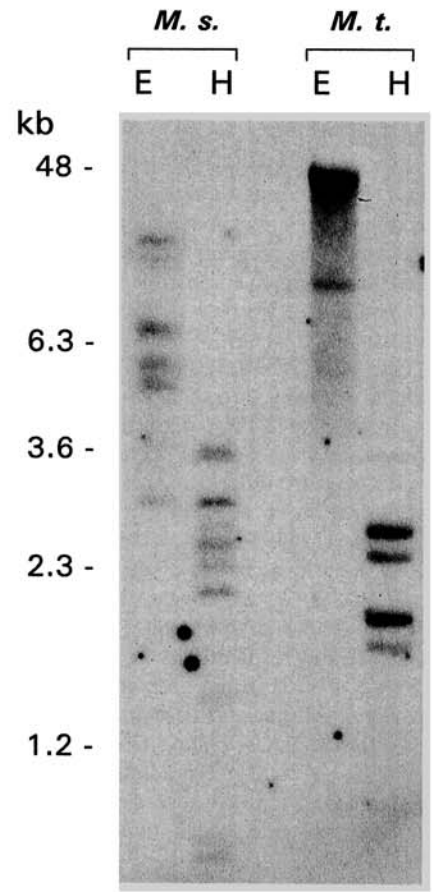

Fig. 1. Analysis of the genomic organization of PR10 in two *Medicago* species. Southern blot was performed with genomic DNA (15 μg/lane) isolated from *Medicago sativa* ssp. *sativa* cv. Nagyszénàsi (*M.s.*) and *M. truncatula* cv. 108-1 (*M.t.*) and digested with *Eco*RI (E) or *Hin*dIII (H). Blot was probed with C7 cDNA clone and the washing conditions of the membrane was 0.1× SSC, 65°C.

Comparison of the deduced amino acid sequence to the sequences available from the data banks revealed significant similarity to proteins of the PR-10 class (Van Loon et al. 1994), ranging from approximately 40% of identity (parsley sequences [Sommsich et al. 1988]) to 81% identity with the pI49 sequence from pea (Fritensky et al. 1988), which has led us to conclude that the C7 polypeptide does belong to the class 10 PR protein as defined by van Loon et al. (1994), therefore the C7 clone was renamed *Ms*PR10-1.

A feature of these PR proteins (see for example, Iturriaga et al. 1992; Somssich et al. 1988) is that they are almost invariably part of gene families. Southern hybridization was carried out with total DNA isolated from two *Medicago* species, the tetraploid *M. sativa* ssp. *sativa* cv. Nagyszénàsi and the diploid *M. truncatula* ecotype 108-1. In the tetraploid plant (Fig. 1, *M.s.*) hybridization with the C7 cDNA clone revealed 8 to 10 bands in the *Eco*RI- or *Hin*dIII-digested DNA, respectively. In the diploid species (Fig. 1, *M.t.*) the patterns of hybridization were less complex: 2 bands (one had high molecular weight) or 4 bands, respectively. These data suggest that this PR10 protein is encoded by a multigene family. To test whether several alfalfa PR genes were expressed during the incompatible interaction with *P. syringae* pv. *pisi*, we used the technique described above for extending the incomplete cDNA sequence; in this experiment the specific oligonucleotide corresponded to a more conserved region, encoding the oligopeptide IDAIQSI (Fig. 2). By subcloning of the PCR products 14 clones were isolated. By sequencing and double-checking, six distinct sequences were obtained (Fig. 2). The differences were found in the 5′ UTR (5′ untranslated region) as well as in the deduced coding region and some of the mutations appeared to be silent. Despite the limited size of the PCR-amplified DNA fragments, these data allowed us to conclude that during the incompatible interaction several genes or allelic variants were expressed in the alfalfa leaves. These sequences may represent two groups of genes/allelic variants (Fig. 2). The first group includes three allelic variants, C7 (i.e., *Ms*PR10-1), C18, and C55. The second group (clones C5, C19, C52) is characterized by amino acid changes which occured repeatedly at the same positions (D vs. E, V vs. I, S vs. A).

Expression analysis.

Our preliminary results (Esnault et al. 1993) indicated that in the untreated leaves of *M. sativa* the *Ms*PR10 gene(s) was (were) expressed at a very low level. To determine whether this gene is developmentally regulated, its expression was analyzed in several organs (roots, hypocotyls, cotyledons,

stems, leaves, and flower buds at three developmental stages) by Northern hybridization using the cDNA clone C7 as a probe for blots obtained after electrophoresis of 20 μg total RNA samples. Transcripts accumulated at high levels in roots but were nearly undetectable in all the other organs (Fig. 3). The same conclusions were drawn when poly(A)$^+$ populations (1 μg per lane) were analyzed, except that sometimes a very faint signal was observable in the hypocotyl and leaf RNA samples.

To get more precise information about the expression of this gene in defense reactions elicited in alfalfa leaves, the kinetics of its expression was determined (i) during an incompatible interaction with *P. syringae* pv. *pisi* and (ii) after injection of salicylic acid at 0.1 mM concentration, the involvement of which in the local and systemic defense mechanisms is well documented (Malamy and Klessig 1992; Klessig and Malamy 1994). As shown in Figure 4 (lane C6), *Ms*PR10-1 transcripts levels in the polyadenylated population from the control leaves (i.e., harvested 6 h after infiltration of $MgCl_2$ at 10 mM, the solution used to resuspend the bacterial population) were undetectable. Upon injection of a 30-μl bacterial suspension (10^9 bacteria ml^{-1}) significant accumulation of transcripts already occurred at 3 h postinfection with a maximum level at approximately 24 to 30 h, and the signal level remained high for at least 72 h. Treatment of the leaf cells with salicylic acid resulted in transient accumulation of the *Ms*PR10-1 transcripts within 1 h after injection, which then decreased between 8 and 24 h (Fig. 5).

To determine the expression pattern of the *Ms*PR10-1 in the leaves infiltrated with bacteria leading to an HR response, two approaches were taken: Northern analysis of the infiltrated vs. the noninfiltrated zones (as defined in the Materials and Methods) and in situ hybridization. For the Northern analysis, total RNAs from the infiltrated and noninfiltrated zones, respectively, were extracted 3, 6, 9, and 30 h after infiltration with *P. syringae* pv. *pisi*, and the *Ms*PR10-1 transcript accumulation was monitored by using the C7 cDNA clone as hybridization probe (Fig. 6). The transcripts were barely detectable in the control leaves (C6). In the infiltrated zone with bacteria (I. Z.), transcript accumulation was observed with the same characteristics as that observed with the whole leaf (Fig. 4): significant increase 3 h after bacterium infiltration with a maximum at around 30 h. In the noninfiltrated tissue (N. I. Z.), the transcripts also accumulated but with several hours of delay, as their level was significantly increased only between 3 and 6 h after infiltration.

In situ hybridization was conducted by using the [^{35}S]-labeled anti-sense C7 RNA and the results obtained (Fig. 7) confirmed the specificity of the *Ms*PR10-1 gene expression and its systemic character. As a matter of fact, no silver grains were detectable above the background level seen in the control (Fig. 7A). In the bacterium-treated leaves the *Ms*PR10-1 transcripts were observed, but an unexpected feature of the pathogen-induced PR10-1 expression was its association with the vascular bundles (Fig. 7B). The injected zone was easily

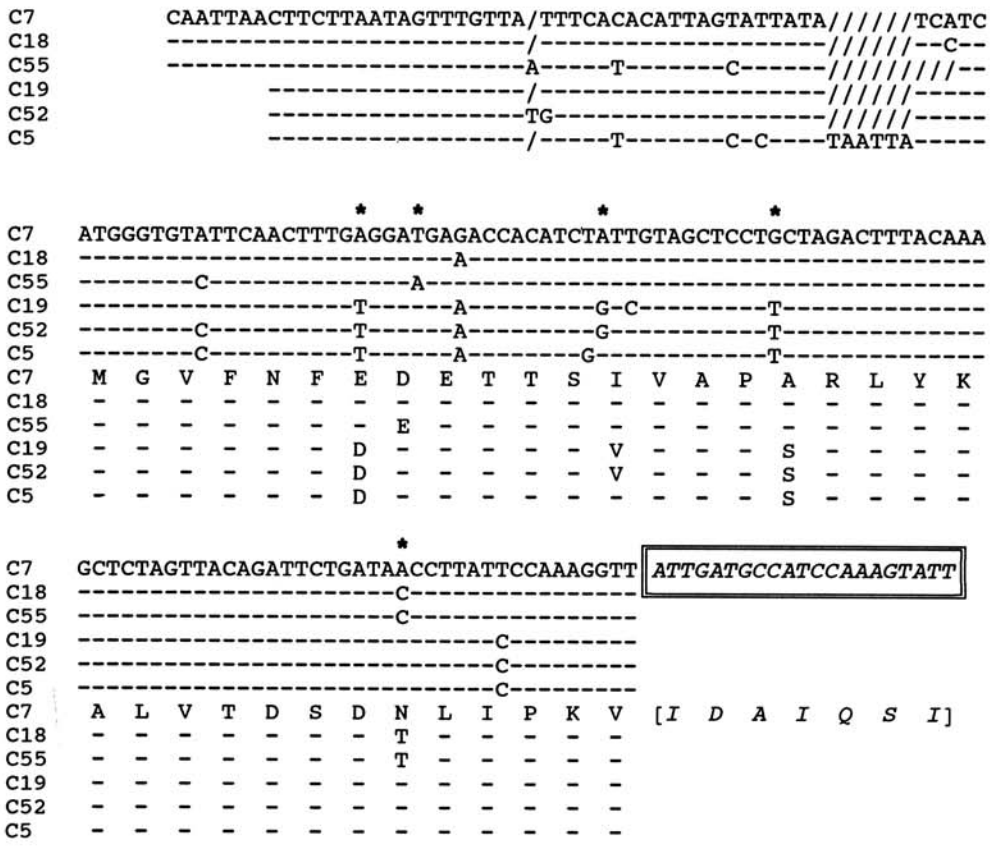

Fig. 2. Alignment of the nucleotide sequences and their deduced amino acids from the C7 cDNA and five other clones obtained by PCR 5′ extension. The minus strand oligonucleotide used for PCR amplification is boxed; the corresponding amino acids are in brackets. Identical nucleotides or amino acids are indicated by dashes; gaps introduced to get the best alignment are indicated by slashes; the * above nucleotides marks nonsilent mutations.

distinguishable from the nontreated region as most of the palisadic cells displayed white fluorescence, probably due to accumulation of phenolic compounds. Silver grains were observable both in the infected and noninfected zones but next to the infected zone strong accumulation of silver grains was observed in the vascular parenchyma. At low magnification (Fig. 7C), accumulation of silver grains was seen in veins far apart, indicating the systemic character of the *Ms*PR10-1 gene expression, at least in the treated leaflet.

DISCUSSION

Here we report the isolation and characterization of a cDNA clone, designated as *Ms*PR10-1, from a cDNA library prepared from mRNA expressed in alfalfa leaf blades undergoing an incompatible interaction with *P. syringae* pv. *pisi* (Esnault et al. 1993). This cDNA encodes a polypeptide belonging to the PR-10 class defined by van Loon et al. (1994): Its sequence identity with representatives of this class ranged from 40% with the parsley sequences to 81% with the pea pI49a clone representing a gene that is expressed in pea pod during pathogenic interaction with *Fusarium solani* (Fristenky et al. 1988). Southern analysis suggested that this PR10 is encoded by a multigene family and, by using a PCR 5′ extension technique (Breda et al. 1996), several mRNAs with slightly different sequence were detectable during the incompatible interaction. However, our data did not allow us to distinguish between different genes and allelic variants. As a matter of fact, because this plant is an allogamous tetraploid species, the existence of allelic variants is expected and our results presented in Figure 2 are in line with this possibility.

One significant characteristic of the *Ms*PR10-1 gene was that it is developmentally regulated: Except in roots, its expression was not detectable in other organs of healthy plants. Similar results were reported with the clone SAM22 (encoding a class 10 PR) isolated from soybean; its expression was nearly undetectable in leaves, it was very low in hypocotyls but high in young roots (Crowell et al. 1992). In roots, constitutive expression of the PR-10 genes was also demonstrated for two clones isolated from pea, pI49a and RH2 isolated from an untreated root cDNA population (Mylona et al. 1994), the coding regions of the two clones sharing 95% identity.

The induction of *Ms*PR10-1 mRNA by *P. syringae* pv. *pisi* was rapid; significant transcript level was already detected 3 h after infection and it accumulated at high level over a period of at least 72 h (Fig. 4). Comparison of the induction kinetics of the *Ms*PR10-1 mRNA with those of genes involved in flavonoid synthesis (for example, chalcone synthase, CHS, chalcone reductase, CHR, chalcone isomerase, CHI, and isofla-

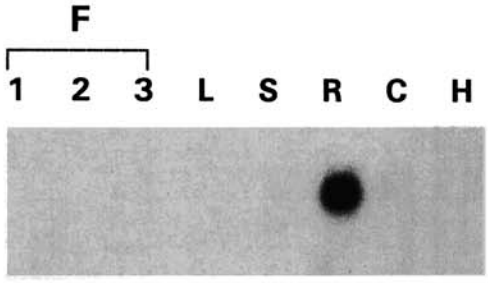

Fig. 3. Northern blot analysis of *Ms*PR10-1 transcripts from different *Medicago sativa* organs. Total RNA (20 µg) from flowers at three different stages of development (numbered 1 to 3, see section Materials and Methods), leaves (L), stems (S), roots (R), cotyledons (C), and hypocotyls (H) were probed with the C7 cDNA clone. Equal loading of RNA populations were checked by staining the membranes with methylene blue.

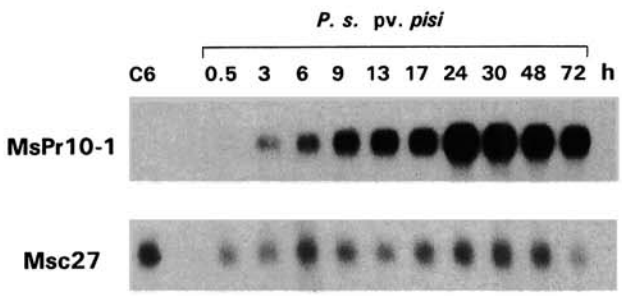

Fig. 4. Time course of the *Ms*PR10-1 transcript accumulation during an incompatible interaction. Northern analysis of poly(A)$^+$ RNA (1 µg/lane) extracted from *Medicago sativa* leaves infiltrated with a suspension of *Pseudomonas syringae* pv. *pisi* and harvested at various times (indicated in hours); lane C6, poly(A)$^+$ RNA from leaves infiltrated with $MgCl_2$ (10 mM) and harvested 6 h later. The blot was probed with ^{32}P-labeled C7 cDNA and unequal loading was checked by using the Msc27 cDNA (Györgyey et al. 1991) considered as constitutive probe.

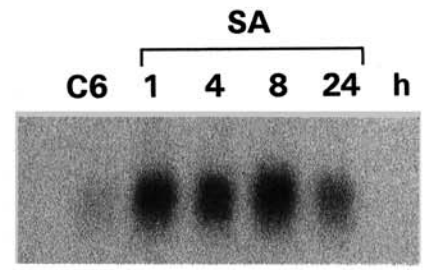

Fig. 5. Time-course of the *Ms*PR10-1 transcript accumulation after infiltration of a solution of salicylic acid at 0.1 mM. Northern analysis of poly(A)$^+$ RNA (1 µg/lane) extracted from *Medicago sativa* leaves harvested at various times (indicated in hours); lane C6, poly(A)$^+$ RNA from leaves infiltrated with $MgCl_2$ (10 mM) and harvested 6 h later. The blot was probed with ^{32}P-labeled C7 cDNA and unequal loading was checked by using the Msc27 cDNA (data not shown).

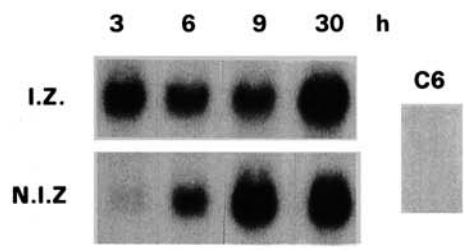

Fig. 6. Accumulation of *Ms*PR10-1 transcripts in the infiltrated (I. Z.) and noninfiltrated zone (N.I.Z.) cut off from leaves after injection of a suspension of *Pseudomonas syringae* pv. *pisi*. Bacteria at 10^9 cells ml^{-1} were infiltrated and the Northern blot of total RNA populations (20 µg/lane), isolated 3, 6, 9, and 30 h after infiltration, respectively, was probed with the C7 cDNA clone; control (C6) consisted of total RNA extracted from leaves harvested 6 h after infiltration with 10 mM $MgCl_2$. Equal loading of RNA populations was checked by staining the membranes with methylene blue.

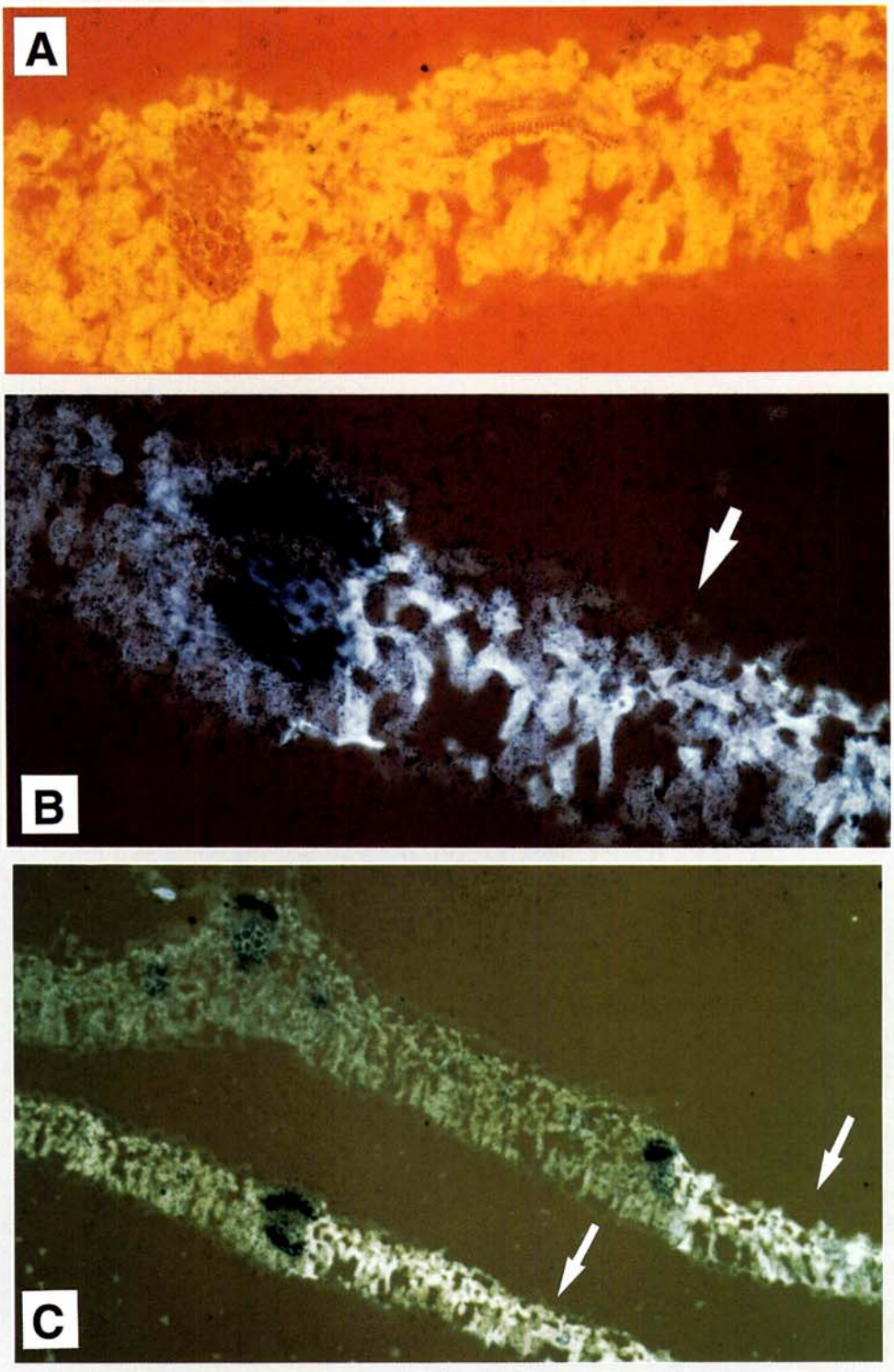

Fig. 7. Localization of *Ms*PR10-1 transcripts in tissue sections of leaf following injection of 10 mM $MgCl_2$ (**A,** control) or a suspension of the bacterium *P. syringae* pv. *pisi* (**B, C**). Sections were prepared 30 h postinoculation and autoradiography following hybridization to in vitro synthesized anti-sense C7 cDNA transcripts. **A,** Control leaves (×300); **B, C,** bacteria infiltrated leaves at two magnifications (×300, B, or ×120, C). A Reichert Polyvar microscope was used in mixed modes of transmission and epifluorescence. In B and C, cells showing bright fluorescence correspond to the infiltrated zone (indicated by white arrows).

vone reductase, IFR), using the same mRNA populations (Esnault et al. 1993; Sallaud et al. 1995) showed that, in our experimental conditions, these genes of different types (PR and flavonoid biosynthesis) were coordinately induced but their kinetics were sufficiently different to suggest that the transduction pathways and/or the regulation steps are probably not identical.

By Northern and in situ hybridizations, we demonstrated the systemic character of the *Ms*PR10-1 expression. Our conclusion is in agreement with the observations made on the interactions of primary leaf buds from parsley with *Phytophthora megasperma* f. sp. *glycinea* (Somssich et al. 1988) and leaves of transgenic tobacco, carrying the gene *AoPR1* promotor-*uid*A fusion, with *Botrytis cinerea* (Warner et al. 1993). By using in situ hybridization, massive accumulation of the parsley PR1 mRNA around the sites infected with spore suspension and strong GUS activity in uninfected cells adjacent to necrotic lesions, respectively, were observed. Our hybridization data (Fig. 7B and C) indicated that *Ms*PR10-1 accumulated in the leaf vascular bundles. As far as the leaf is considered, our results are in agreement with data obtained for other PR protein genes unrelated to the class 10, such as *win*, *PR-1*, exhibiting expression in vascular bundles (Carr et al. 1987; Stanford et al. 1990; Zhu et al. 1993) but in contradiction with the results of Constabel and Brisson (1995). These authors have shown that following infection of transgenic tobacco plants, carrying the promoter PR-10a-*uid*A fusion, with pathogenic *Pseudomonas infestans*, the expression of this chimeric gene was high in all the vegetative organs tested, with the exception of the leaves. However, histochemical GUS staining of the responsive organs (tubers, stolons, stems, and petioles) indicated that PR-10a induction was associated with vascular bundles.

The expression of the *Ms*PR10-1 gene in vascular bundles was found not only adjacent to the infected zone but also distant from the infection site and its responsiveness to salicylic acid treatment suggested its systemic character. We are currently studying the possible involvement of this (these) gene(s) in the systemic acquired resistance.

MATERIALS AND METHODS

Growth of bacteria, plants, and treatment of plants.

All of these conditions were previously described (Esnault et al. 1993). In experiments where the expression in the infiltrated and noninfiltrated zones was studied, the sharply localized and distinguishable infiltrated area after syringe-infiltration allowed us to easily distinguish these two parts. The infiltrated zone corresponded to the infiltrated area per se plus 1 to 2 mm of the surrounding tissue, whereas the noninfiltrated zone was the remainder part of the leaf blade.

Preparation of RNA and blot analysis, construction and screening of cDNA library, subcloning, and DNA sequencing.

As previously described (Esnault et al. 1993). For quantitation of the loaded RNA one of two methods was used, depending on the analyzed population. For Northern blots of polyadenylated RNA, the membranes were probed with Msc27 cDNA which was used as a constitutively expressing gene probe (Györgyey et al. 1991). For total RNA populations, loading of the gel was checked by staining the membrane with methylene blue as described (Sallaud et al. 1995).

DNA extraction and Southern blot analysis.

As described by Sallaud *et al.* (1995).

PCR amplification.

PCR amplification was carried out in a Crocodile Thermocycler (Appligène, Illkirch, France). Typically, 1 μg of cDNA ligated to the *Eco*RI adaptor (Amersham, Les Ulis, France) was combined with 70 pmol of each oligonucleotide primers in a 50 μl reaction mixture (10 mM Tris HCL, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$; each dNTP at 200 μM; 0.1% Triton X-100; 0.2 mg ml^{-1} bovine serum albumin) followed by addition of 0.2 units of *Taq* DNA polymerase (Appligène) and 50 μl of mineral oil. Reactions conditions (standard amplifications) were 5 min at 95°C and 25 cycles of 95°C 1 min, 53°C 2 min, 72°C 2 min. Our PCR approach was based on the use of one primer specific for the adaptor (upper primer, 5′ → 3′) and another specific for a sequence within the cDNA (lower primer, 3′ ← 5′, complementary to the + strand) (Breda et al. 1996).

In situ hybridization.

It was carried out according to Grosskopf et al. (1993). Control or infected leaflets were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M sodium phosphate buffer at 4°C for 5 h, dehydrated in ethanol/butanol series and transferred in increasing concentrations of butanol-saturated paraplast at 30°C for 4 h, then 42°C overnight and in pure paraplast at 60°C for 2 days. The embedded tissues were solidified at room temperature and sectioned into 8-μm-thick slices, fixed on polylysine coated glass slides. Paraplast was removed with xylene and sections were rehydrated through a decreasing serial dilution of ethanol. Before hybridization, the sections were incubated in a pronase solution (10 μg ml^{-1} in phosphate buffer, pH 7.5) at 37°C for 15 min. To diminish the background, the slides were dipped into an acetylation solution (0.1 M triethanolamine, 2.5% anhydrous acetic acid) for 10 min at room temperature, then washed in water and dehydrated in an increasing serial dilution of ethanol.

Hybridizations were conducted with an anti-sense strand probe, obtained by transcription with the T_7 RNA polymerase kit (Boehringer) from a linearized pBluescript SK^+ harboring a fragment from the C7 cDNA clone corresponding to the 3′ end delimited by the *Bam*HI/*Eco*RI sites, and labeled with [^{35}S]UTP (37 TBq $mmol^{-1}$). After washing, the slides were coated with the NTB-Z (Kodak) photographic emulsion and exposed at 4°C for 3 weeks.

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