Pathological and Molecular Characterizations of Alfalfa Interactions with Compatible and Incompatible Bacteria, *Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*

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We report on the interactions of alfalfa with *Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*. A hypersensitive response was observed when leaves were infiltrated with *P. s.* pv. *pisi*, which remained strictly limited to the injected zone. The compatible interaction with *X. c.* pv. *alfalfae* was characterized by water-soaking symptoms and the spreading of the bacterium into the leaf blade. Analyses of transcript accumulation were conducted with cDNAs encoding enzymes involved in phytoalexin synthesis: chalcone synthase (CHS), chalcone isomerase (CHI), and isoflavone reductase (IFR). In incompatible interactions the maximum accumulation of the CHS, CHI, and IFR transcripts was observed 6 hr postinfection. In the compatible interaction, the induction of these transcripts was delayed until 25–30 hr postinfection, and the level of their accumulation was considerably lower. Extending this molecular analysis to the root system showed that the reaction of roots during an incompatible interaction was quite comparable to that of leaves. To complete these analyses, expression of genes encoding pathogenesis-related (PR) proteins in leaves was also analyzed by polymerase chain reaction. High-level accumulation of a 0.8-kb transcript encoding a PR protein was observed 6 to 30 hr postinfection in the incompatible interaction.

Additional keywords: chalcone isomerase gene expression; chalcone synthase gene expression; isoflavone reductase gene expression; incompatible/compatible interactions; *Medicago sativa*; pathogenesis-related protein gene expression; phytopathological reactions.

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The chalcone synthase nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession numbers X68106 and X68107.

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Intensive study over the past two decades has led to an understanding of many of the molecular events in early plant-*Rhizobium* communication leading to nodule organogenesis. Nodule induction and development as well as invasion of plant cells by rhizobia do not seem to provoke host defense responses, which might suggest that *Rhizobium* has evolved a mechanism to circumvent the host defense response (Verma and Nadler 1984). The formation of a subcellular compartment housing the bacteria inside an infected cell is a fundamental step for a successful infection. The failure to form this membrane compartment or its disintegration renders the association ineffective, as has been shown for mutants of *Bradyrhizobium japonicum* (Werner *et al.* 1985) and *R. meliloti* (Putnoky *et al.* 1988). In these cases, alterations of the peribacteroid membranes and other morphological changes are reminiscent of those evoked by host defense responses. This suggests that the cellular compartmentalization could be part of the mechanism evolved by rhizobia to escape the host defense system. Such a mechanism might also involve the modulation of plant defense gene expression by some bacterial gene products. Another hypothesis is that rhizobia might be able to escape the elicited plant defense mechanism by, for example, detoxifying the phytoalexins produced.

Cultivated *Medicago sativa* is a host of a wide range of fungal pathogens (Graham *et al.* 1979), and several reports have suggested that the accumulation of the isoflavonoid medicarpin may be involved in the resistance of the plant to these pathogens (Higgins 1972; Latunde-Dada and Lucas 1985; Dixon 1986). Significant progress has been made in defining specific genes encoding phytoalexin-biosynthetic enzymes in cultured alfalfa cells (Jorrin and Dixon 1990; Gowri *et al.* 1991; Harrison *et al.* 1991; Paiva *et al.* 1991). In spite of the fact that bacterial diseases (such as wilt induced by *Corynebacterium insidiosum*, leaf spot by *Xanthomonas campestris* pv. *alfalfae*, and stem blight by *Pseudomonas syringae*) may be severe, little is known about the molecular basis of bacterial interactions with this plant.

To study how the defense mechanism can be elicited

and/or controlled during nodulation, a fundamental condition had to be fulfilled: the availability of markers specifically involved in a defense reaction. We developed a system based on the reaction of alfalfa to infection by *P. s.* pv. *pisi* (nonpathogenic to alfalfa: an incompatible interaction) and infection by *X. c.* pv. *alfalfae* (a compatible interaction). We followed the course of events at the molecular level by using several probes derived from genes implicated in the defense reaction.

The defense mechanisms elaborated by plants involve the production of phytoalexins, the deposition of lignin-like phenolic material, the synthesis of hydroxyproline-rich glycoproteins and a range of so-called pathogenesis-related (PR) proteins, including the hydrolytic enzymes chitinase and 1,3-β-glucanase (reviewed by Lamb *et al.* 1989). In this paper accumulation of transcripts from genes encoding enzymes of the phenylpropanoid branch pathway—chalcone isomerase (CHI), chalcone synthase (CHS), and isoflavone reductase (IFR)—as well as genes encoding PR proteins were studied in leaves and roots exposed to pathogenic bacteria in compatible and incompatible interactions.

RESULTS

Phytopathological analysis.

Screening of bacteria for compatible and incompatible interactions with alfalfa. In the first step of this study, we determined phenotypically the outcome of interactions of *M. sativa* with pathogenic bacteria, with respect to either a compatible or an incompatible interaction. As shown in Figure 1, infiltration of leaves with *X. c.* pv. *alfalfae* gave

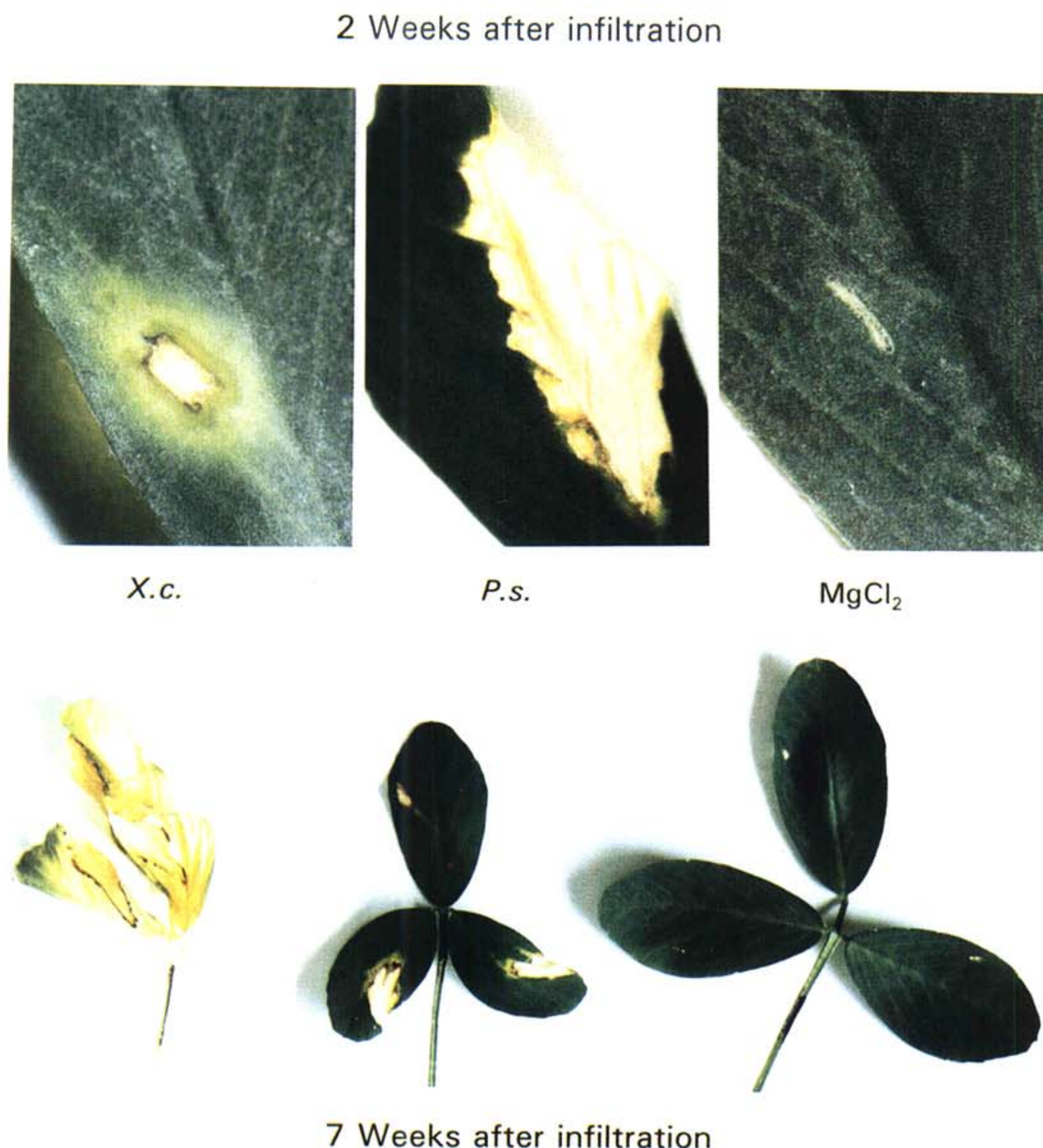

Fig. 1. Reactions of alfalfa leaves to injection with *Xanthomonas campestris* pv. *alfalfae* (*X.c.*) and *Pseudomonas syringae* pv. *pisi* (*P.s.*). Leaf blades were injected with bacterial suspensions of 10^9 cfu ml^{-1} in 10 mM $MgCl_2$; control leaves were infiltrated with 10 mM $MgCl_2$. The photographs were taken 2 and 7 weeks after injection. In order to clearly show the water-soaking symptoms induced by *X. c.* pv. *alfalfae* (as observed 2 weeks after infiltration), the volume of the injected suspension was deliberately smaller than that of the *P. s.* pv. *pisi* suspension.

rise to the symptoms expected in a compatible reaction, i.e., spreading water-soaked lesions. Long-term effects were indicated by yellowing and wilting of the whole leaf.

Incompatible bacteria were identified by screening different strains of *X. campestris* for their ability to induce a hypersensitive response on alfalfa leaves. Leaf necrosis was observed 4 to 5 days after injection with *X. c.* pv. *vignicola* or *X. c.* pv. *holcicola* (data not shown). More rapidly developing symptoms characteristic of an incompatible reaction were obtained with *P. s.* pv. *pisi*. Leaf tissue infiltrated with 10^9 cells per milliliter began to collapse 5–6 hr after inoculation, and necrosis of the injected

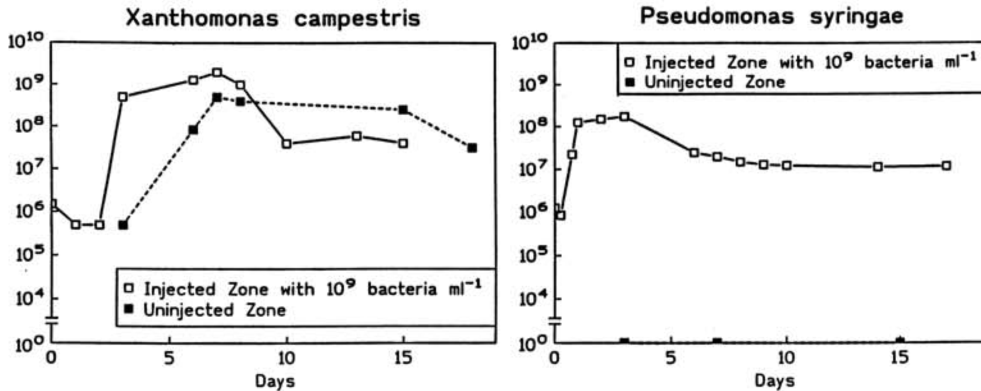

Fig. 2. Multiplication of *Xanthomonas campestris* and *Pseudomonas syringae* in alfalfa leaves. Leaf blades were injected with a suspension of *X. c.* pv. *alfalfae* or *P. s.* pv. *pisi* at 10^9 cfu ml^{-1} in 10 mM $MgCl_2$; leaf disks were punched from the injected or the noninjected zone and ground in 10 mM $MgCl_2$. The number of colony-forming units per disk was determined by serial dilution and plating and expressed per square centimeter.

Fig. 3. A and **B,** Induction of chalcone synthase (CHS) (**A**) and chalcone isomerase (CHI) (**B**) messages in alfalfa leaves upon infection with *Pseudomonas syringae* pv. *pisi* determined by a time-course experiment (dot blots) and Northern blots. Polyadenylated mRNAs, extracted from leaves 1.5, 4, 6, and 17 hr after infection or (in control leaves, designated C6) 6 hr after $MgCl_2$ infiltration, were dot-blotted (5–500 ng per dot); mRNAs from treated leaves 6 hr after infection (P6) or control leaves 6 hr after infiltration (C6) were used for Northern blotting (0.5 μg of RNA per slot). Probing was carried out by means of heterologous CHS and CHI cDNAs (from *Phaseolus vulgaris*). **C,** Treatment/control ratios obtained from densitometric readings of the radiograms; the 6-hr control value was used as a reference point, considered equal to 1.

zone was clearly achieved within 24 hr (data not shown). The necrosis remained restricted to the infiltrated area up to 7 weeks after infiltration (Fig. 1).

Growth of bacteria in leaves. The growth of *X. c.* pv. *alfalfae* and *P. s.* pv. *pisi* was examined for up to 15 days in both injected and noninfiltrated zones, defined in Materials and Methods. As shown in Figure 2, *X. c.* pv. *alfalfae* grew within the infiltrated area, its population increasing by a factor of 10^3 after 6 days, and it began to spread in the leaf blade from day 4 onwards. The multiplication of *P. s.* pv. *pisi* in the injected zone was lower (it grew by a factor of 10^2), and it appeared that the bacterial population was strictly limited to the infiltrated area, as expected for incompatible pathogenic bacteria. Therefore, the incompatible interaction of *P. s.* pv. *pisi* and alfalfa was chosen for further study.

Molecular analysis.

CHI, CHS, and IFR transcript accumulation during incompatible and compatible interactions. In order to gain a preliminary understanding of the possible involvement of the alfalfa phenylpropanoid pathway in the incompatible interaction we monitored this process by using several heterologous cDNA probes from *Phaseolus vulgaris* (kindly supplied by C. J. Lamb, Salk Institute). A time-course experiment was carried out with leaves infiltrated with either *P. s.* pv. *pisi* or $MgCl_2$, and the patterns of CHI and CHS transcript accumulation were determined by Northern analysis. As shown in Figure 3, the accumulation of CHI and CHS transcripts (1- and 1.4-kb, respectively) was detectable as early as 90 min after bacterial injection and seemed to be maximal at around 6 hr. Quantitative determination of transcript accumulation by means of a densitometer revealed (Fig. 3C) that 6 hr after injection with *P. s.* pv. *pisi* or $MgCl_2$, CHS and CHI transcripts were approximately 85 and 20 times more abundant, respectively, in the incompatible interaction than in the control leaves.

To more precisely evaluate the level of CHS transcript, homologous CHS gene probes were isolated from a cDNA library constructed in λgt10, from poly(A)$^+$ RNA extracted from leaves 4 hr after infiltration with *P. s.* pv. *pisi*. A 21-mer oligonucleotide corresponding to a highly conserved CHS region (AAA/C-GAA/C-TTA/C-GCC/T-AAC/T-AAC encoding AKDLAENN; see Figs. 4 and 5) was used to obtain five positive clones from 13,000 independent plaques. Two clones, designated MsCHSI and MsCHSII, were sequenced; MsCHSI corresponded to a full-length cDNA, whereas MsCHSII lacked the 5′ coding region, beginning 368 bp downstream from the first ATG identified in MsCHSI (Fig. 4). The coding regions and the 3′ untranslated regions of these two clones showed 97.2 and 83.4% homology at the nucleotide level, respectively. At the amino acid level, MsCHSI and MsCHSII differed only by a missing codon in MsCHSII and by a single amino acid change (Thr instead of Ala in CHSII). Alignment of the deduced amino acid sequence of MsCHSI with other CHS sequences (Fig. 5) showed a very high conservation of all CHS sequences. The alfalfa CHS sequence appeared to be more homologous to the bean sequence (88.9% homology) than to any other, as confirmed by a dendrogram analysis (Clustal program,

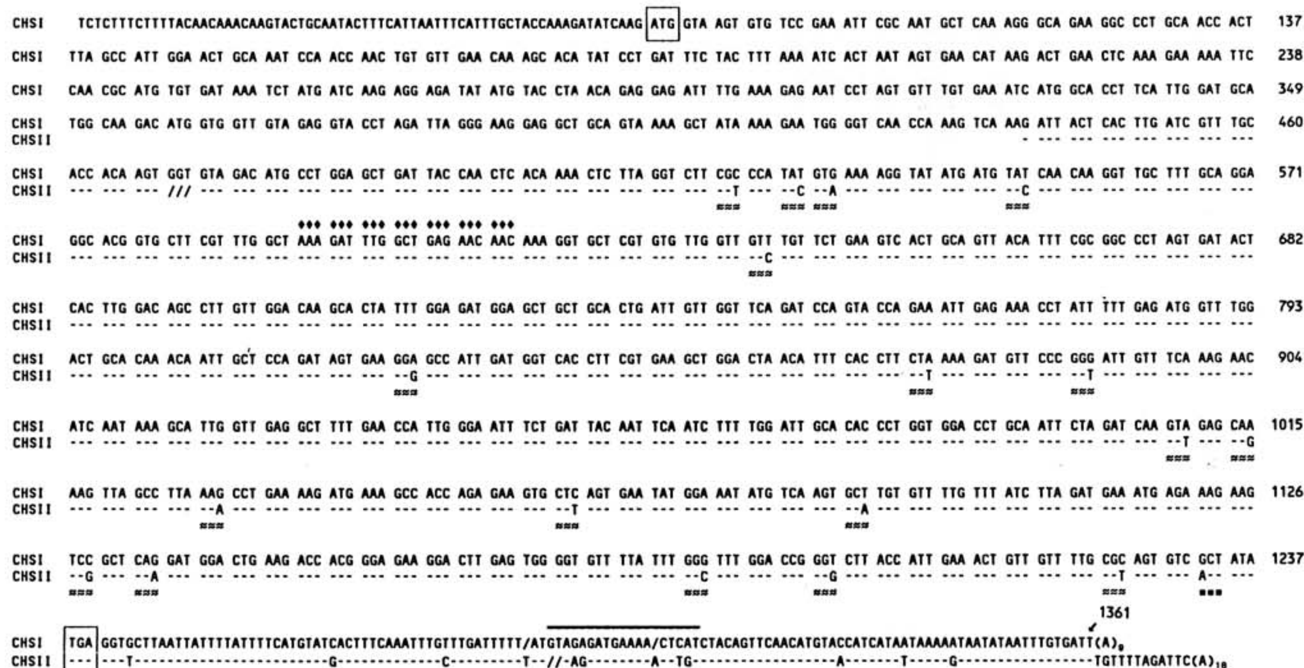

Fig. 4. Nucleotide sequences of MsCHSI and MsCHSII, the two chalcone synthase (CHS) cDNAs from alfalfa. Identical nucleotides are represented by dashes, and deletions by slashes; silent mutations are underlined with ≈≈≈, and amino acid changes with ■ ■ ■. The putative translation start and stop codons are boxed. The position of the oligonucleotide used as a probe is indicated by ♦ ♦ ♦. The positions of the oligonucleotides Oligo CHSI and Oligo CHSII are indicated by overlines and underlines, respectively.

PC/Gene) (data not shown).

For comparison of the incompatible and compatible interactions, polyadenylated RNA populations from leaves harvested 1.5 to 30 hr after infection with *P. s.* pv. *pisi* or *X. c.* pv. *alfalfae* were hybridized in Northern blots with two cDNAs: clones MsCHSI and IFR (clone from *M. sativa,* provided by R. A. Dixon). MsCHSI represents one of the initial steps of the phenylpropanoid branch pathway, while IFR is specific for the isoflavonoid branch and catalyzes one of the last steps in the synthesis of the alfalfa phytoalexin medicarpin (Paiva *et al.* 1991). As shown in Figure 6A and C, there was a rapid accumulation of CHS and IFR messages by 90 min after injection with *P. s.* pv. *pisi.* The transcript accumulation reached its maximum level 6 hr after injection and then decreased. The CHS transcripts leveled off rapidly, but the IFR transcripts remained at a relatively high level throughout the assay period. These results indicate that the isoflavonoid branch pathway is preferentially induced during the incompatible interaction with *P. s.* pv. *pisi.* During the compatible interaction (Fig. 6B and D), significant accumulation of CHS and IFR transcripts was delayed until 25–30 hr after inoculation, with hybridization signals considerably lower than those observed 6 hr after treatment with the incompatible bacteria.

In order to determine if the two alfalfa CHS clones might be differentially expressed, we generated oligonucleotide probes that can differentiate the two clones. The specificity of the designed oligonucleotides for MsCHSI and MsCHSII was ascertained by a dot blot experiment in which no cross-hybridization was detected under our experimental conditions (Fig. 7). These probes, along with the MsCHSI cDNA coding region as a control, were then used to analyze CHS expression in leaves and roots upon infection with *P. s.* pv. *pisi* or *X. c.* pv. *alfalfae* and in untreated flowers as well. Based on the kinetics of the bacterial interactions (Fig. 6), two time points were chosen: 6 hr for the *P. s.* pv. *pisi* interactions and 30 hr for the *X. c.* pv. *alfalfae* interactions. Two controls were necessary for the root treatment with bacteria (see Materials and Methods): untreated roots, which were dipped for 6 hr in a nutrient solution (U6), and roots that were gently scratched before they were dipped in the same solution (C6). Figure 8 shows that CHS was weakly expressed in roots and flowers and barely detectable in leaves of control plants, whereas wounding of the root led to a net in-

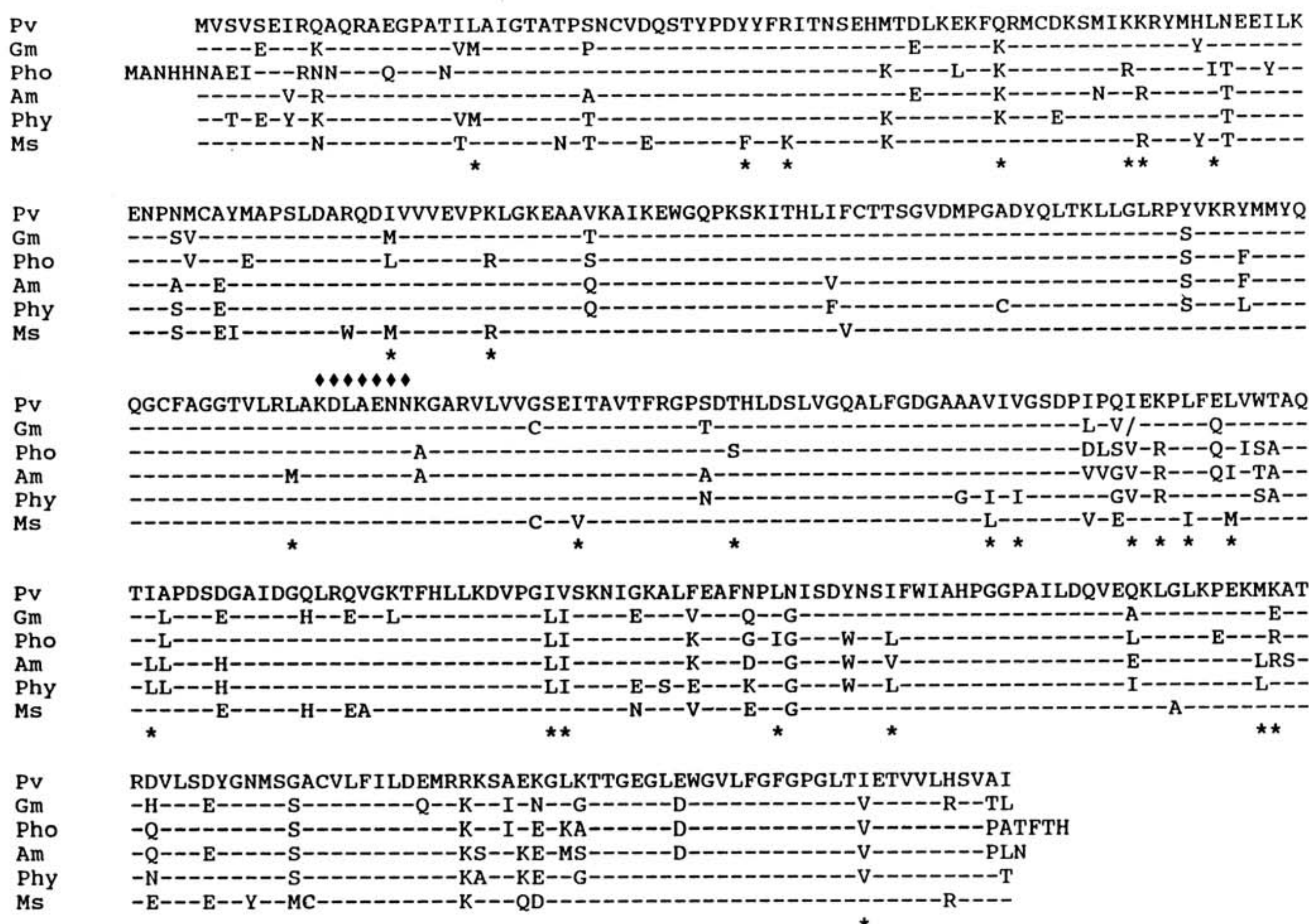

Fig. 5. Alignment of the amino acid sequence deduced from MsCHSI with the chalcone synthase (CHS) sequence from other species: Pv = CHS1 from *Phaseolus vulgaris* (Ryder *et al.* 1987); Gm = CHS gene 1 from *Glycine max* (Akada *et al.* 1987); Pho = CHS gene from *Petroselinum hortense* (Herrmann *et al.* 1988); Am = CHS from *Antirrhinum majus* (Sommer and Saedler 1986); Phy = CHS gene A from *Petunia hybrida* (Koes *et al.* 1989); Ms = MsCHSI from *Medicago sativa.* Identical amino acids are represented by dashes, equivalent amino acids (F/Y; K/Q/R; S/T; V/I/L/[M]) by asterisks. The position of the oligo probe used for the screening is indicated by ♦ ♦ ♦.

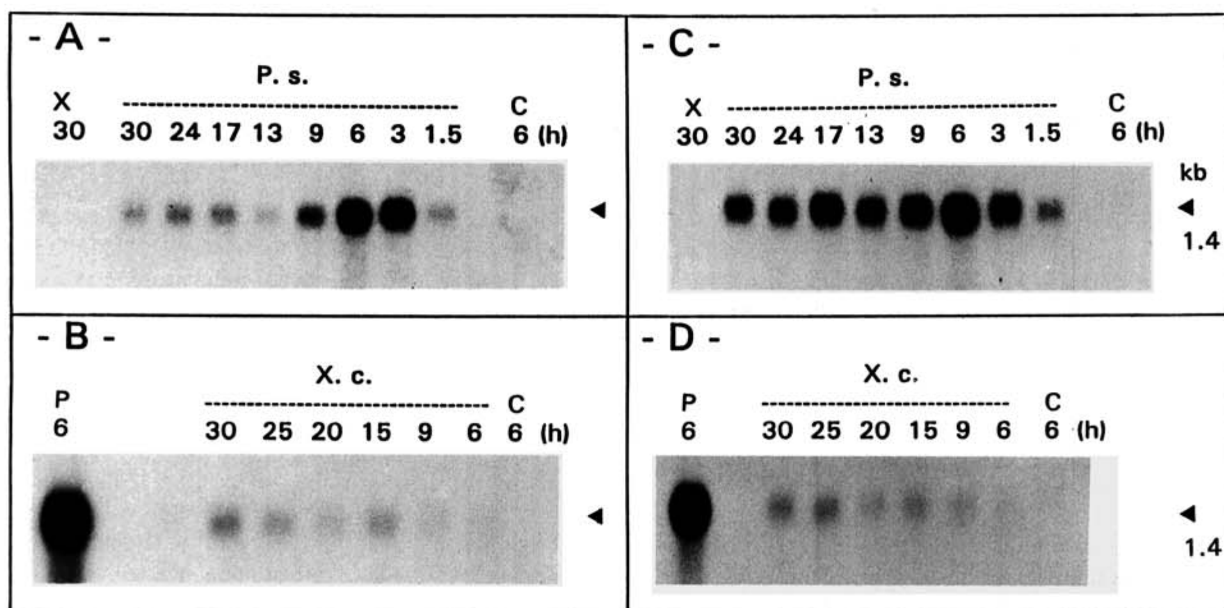

Fig. 6. Analysis of expression of chalcone synthase (CHS) (**A** and **B**) and isoflavone reductase (IFR) (**C** and **D**) in alfalfa leaves upon treatment with an incompatible bacterium (*Pseudomonas syringae* pv. *pisi*) or a compatible bacterium (*Xanthomonas campestris* pv. *alfalfae*). Polyadenylated RNA isolated from leaves harvested 1.5 to 30 hr after infection with *P. s.* pv. *pisi* (**A** and **C**) or *X. c.* pv. *alfalfae* (**B** and **D**) were loaded (1 μg per lane) and probed (after electrophoresis and blotting) with alfalfa CHS and IFR cDNAs. Controls (C6) consisted of mRNA extracted from leaves harvested 6 hr after infiltration with 10 mM $MgCl_2$. Northern blots in **A** and **C** include, for comparison, mRNA extracted from leaves treated with *X. c.* pv. *alfalfae* (X30, harvested 30 hr after infection), and those in **B** and **D** include mRNA from leaves treated with *P. s.* pv. *pisi* (P6, harvested 6 hr after infection).

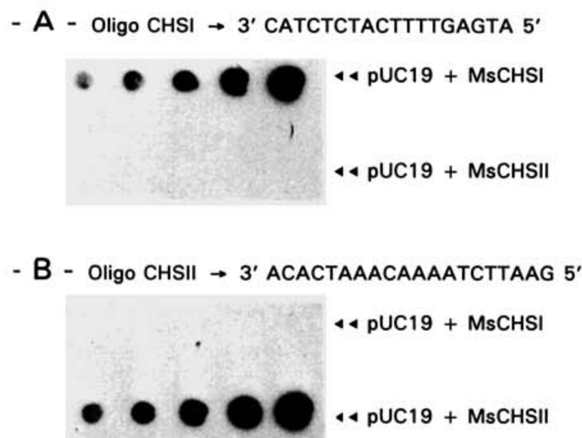

Fig. 7. Dot blot experiment to evaluate the "specificity" of Oligo CHSI (**A**) and Oligo CHSII (**B**), two oligonucleotides designed from the 3′ noncoding region of two chalcone synthase cDNAs from alfalfa. The cDNA solutions were adjusted in order to spot (onto two different membranes) from 75 down to 4.7 fmol (2× dilution at each step) of MsCHSI and MsCHSII inserts; each membrane was hybridized with either Oligo CHSI (**A**) or Oligo CHSII (**B**).

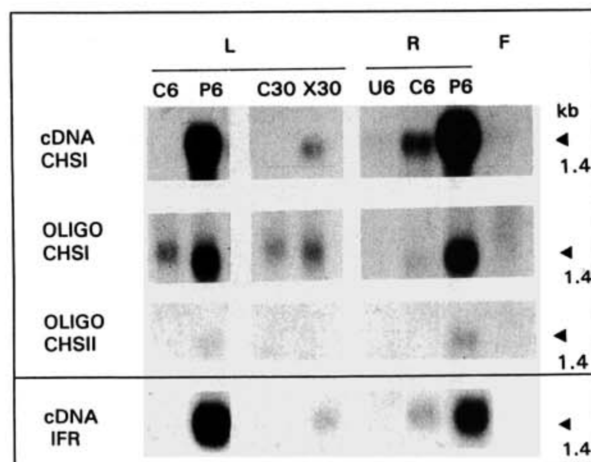

Fig. 8. Autoradiograms of Northern blots showing the differential expression of alfalfa chalcone synthase (CHS) and isoflavone reductase (IFR). Analyses were carried out on roots (R) and leaves (L) harvested from whole plants subjected to pathogenic interactions with *P. s.* pv. *pisi* for 6 hr (P6) or *X. c.* pv. *alfalfae* for 30 hr (X30). Flower messenger RNAs (F) were extracted from untreated plants. The controls were leaves harvested 6 and 30 hr after $MgCl_2$ infiltration (C6 and C30, respectively) and roots that were unwounded or were gently scratched with sandpaper before being dipped in a nutrient solution for 6 hr (U6 and C6, respectively). Probing was done with the full-length MsCHSI cDNA (1 μg of mRNA per slot), the oligo CHSI or oligo CHSII (3 μg of mRNA per slot), or the full-length IFR cDNA (1 μg of mRNA per slot).

crease in transcript accumulation. The increase in the CHS transcript level in roots infected by *P. s.* pv. *pisi* indicates that there is no organ specificity, as leaf and root showed quite similar responses. Each treatment preferentially induced accumulation of MsCHSI mRNAs and not the MsCHSII transcripts.

As also shown in Figure 8, the pattern of IFR expression was similar to that of CHS during both the incompatible interaction (with a maximum at around 6 hr) and the compatible interaction.

Induction of PR protein transcripts in leaves after bacterial infection. In order to study the kinetics of the appearance of the PR protein transcript upon treatment with *P. s.* pv. *pisi* or *X. c.* pv. *alfalfae*, a time-course experi-

ment was carried out with leaves infiltrated with bacteria or $MgCl_2$. Since no alfalfa sequence for these genes was available, antisense oligonucleotides designed from conserved regions of PR proteins expressed in leguminous plants (such as pea and soybean; see Materials and Methods) were used to synthesize polymerase chain reaction (PCR) fragments of 320 nucleotides (Fig. 9). This clone was used for probing Northern blots corresponding to PR protein expression in the incompatible and compatible interactions. Figure 10 shows that only one PR protein transcript (0.8 kb) was expressed in the incompatible reaction. The transcript level was already high 6 hr postinfection, and its accumulation further increased up to 30 hr postinfection. In contrast, this transcript was practically undetectable in the compatible interaction, even 30 hr after infection.

DISCUSSION

In this study we developed a system for the functional analysis of defense response genes in alfalfa. The initial step in establishing this system was to identify bacterial strains reproducibly eliciting responses characteristic of resistance reactions. We found that several strains of *X. campestris* (pv. *holcicola* and pv. *vignicola*) and *P. syringae* elicited a hypersensitive response. For instance, the *P. s.* pv. *pisi* isolate, which we then studied in more detail, very rapidly induced a localized necrotic lesion, within 24 hr, which is typical of a hypersensitive response (Klement 1982). Injected at a concentration of 7×10^6 cfu ml^{-1} (data not shown) or 10^9 cfu ml^{-1}, it was unable to spread into the leaf blade. In contrast, *X. c.* pv. *alfalfae* multiplied 10^3-fold within 6 days after infiltration and led to water-soaked lesions characteristic of the spreading of this bacterium into the leaf blade, indicating a compatible interaction.

In conjunction with the identification of the incompatible *P. syringae* and the compatible *X. campestris* isolates, we analyzed the expression of two genes (CHS and IFR) involved in the synthesis of medicarpin, which might be one of the major potential antimicrobial phytoalexins in *M. sativa*, at least against fungi (Higgins 1972; Latunde-Dada and Lucas 1985). A number of studies have correlated the induction of the expression of these genes with a resistance response (reviewed by Dixon and Harrison 1990; Paiva *et al.* 1991). Our current results showed a strong correlation of a rapid accumulation of CHS and IFR mRNAs with a resistance response. Increased mRNA levels for these genes showed differential timing in compatible and incompatible interactions. Injecting leaves with *P. s.* pv. *pisi* or dipping roots in a suspension of this bacterium led rapidly (within 90 min) to a switch in the pattern of accumulation of these mRNA species. The massive, albeit transient, accumulation of these transcripts reached its maximum level 6 hr postinfection. In contrast, in the compatible interaction (with *X. c.* pv. *alfalfae*) the transcripts of these defense genes did not accumulate until 24–30 hr after infiltration, and the hybridization signals were considerably lower.

For CHS, similar results have been obtained in other plant-pathogen systems (reviewed by Lamb *et al.* 1989; Dixon and Harrison 1990). For example, differential transcript accumulation has been observed in bean hypocotyls infected with the pathogenic fungus *Colletotrichum lindemuthianum* (Bell *et al.* 1986) and in hypocotyls (Esnault *et al.* 1987), roots (Habereder *et al.* 1989), and leaves (Dhawale *et al.* 1989) of soybean cultivars treated with *Phytophthora megasperma* f. sp. *glycinea* zoospores or *P. s.* pv. *glycinea*. In the case of IFR, the transcript levels appeared to be highly increased in elicited alfalfa cells, but low levels were found in healthy plant parts (Paiva *et al.* 1991).

Our kinetic data suggest coordinated expression of the CHS and IFR genes in alfalfa during an incompatible interaction. As CHS is one of the first and IFR one of the

Fig. 9. Nucleotide sequence and deduced amino acid sequence from polymerase chain reaction (PCR) amplification products corresponding to pathogenesis-related proteins. PCR amplification products, synthesized from the cDNA population (used for preparation of the cDNA library) by using oligonucleotides designed from conserved regions (underlined bold letters), were subcloned and sequenced. One of these sequences is shown, together with the amino acid sequence of the pea clone pI49 (Fristensky *et al.* 1988). Identical amino acids are represented by dashes.

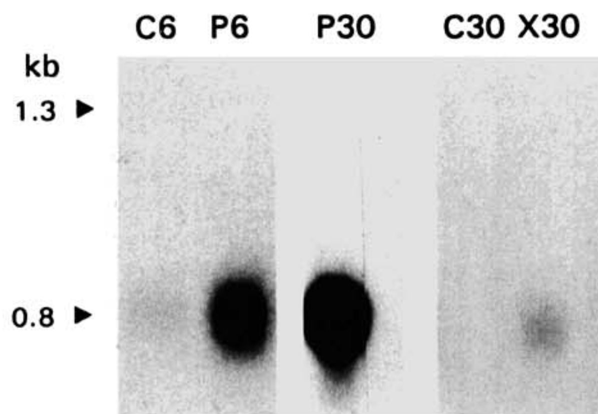

Fig. 10. Probing of the pathogenesis-related (PR) protein expression in alfalfa leaves infected with *Pseudomonas syringae* pv. *pisi* or *Xanthomonas campestris* pv. *alfalfae*. The Northern blots correspond to the incompatible interaction (P6 and P30: mRNA from leaves 6 and 30 hr after infection by *P. s.* pv. *pisi*) and the compatible interaction (X30: 30 hr after infection by *X. c.* pv. *alfalfae*); C6 and C30 are the control leaves, harvested 6 and 30 hr, respectively, after $MgCl_2$ infiltration. Probing was done with the DNA clone obtained from the subcloning of polymerase chain reaction products (see Fig. 9). The amount of deposited mRNAs was 3 µg per slot.

last enzymes committed to the synthesis of medicarpin, this would mean that all the genes involved in this pathway are likely to be coordinately regulated. Previously, such a coordination had been suggested for several genes involved in the early and common stages of the phenylpropanoid pathway, for genes encoding phenylalanine ammonia-lyase (PAL), 4-coumarate:coenzyme A ligase, CHS, and CHI (reviewed by Dixon and Harrison 1990). Our results suggest that, as shown for many other plants, isophenylpropanoid biosynthesis may be an important component of the defense response of *M. sativa*.

We also analyzed the accumulation of transcripts for another putative defense gene, i.e., one encoding PR protein molecules. RNA blot analyses, using a hybridization probe corresponding to molecular species belonging to the group I disease resistance response genes of pea (clone pI49 or pI176) (Fristensky *et al.* 1988), demonstrated that the level of a 0.8-kb mRNA increased during the incompatible interaction of leaves with *P. s.* pv. *pisi* but not during the compatible interaction with *X. c.* pv. *alfalfae*. The kinetics of its accumulation (Fig. 10) is in accordance with the results obtained by Daniels *et al.* (1987) for clones pI49 and pI176 expressed in pea leaves infected with *P. s.* pv. *pisi*. The induction kinetics of the PR protein gene of alfalfa showed a maximum accumulation of transcripts at around 30 hr, in contrast to the kinetics observed for CHS and IFR, in which the maximum accumulation occurred at around 6 hr. Thus, our results indicate that differential regulation of various defense genes also occurs in *M. sativa*.

In several legumes (bean, soybean) several enzymes involved in the isoflavonoid pathway are encoded by small (three- to eight-member) gene families (reviewed by Templeton and Lamb 1988; Lamb *et al.* 1989; Dixon and Harrison 1990). This is particularly the case for PAL, the first enzyme committed to the synthesis of all the phenylpropanoid compounds, and for CHS, the first enzyme specific for the formation of flavonoids and isoflavonoids. Qualitative and quantitative differences in the expression patterns of the members of the family (Liang *et al.* 1989; Paiva *et al.* 1991) were observed when the genes were induced by different environmental or developmental stimuli. In the case of bean CHS, at least five different genes were induced in elicitor-treated cells (Ryder *et al.* 1987). In alfalfa, identification of the translation products of defense-related mRNAs, induced in elicitor-treated cells, showed that mRNAs encoding CHS isopolypeptides appeared to be among the most abundant transcripts induced (Dalkin *et al.* 1990). Alfalfa CHS might also be encoded by a multigene family, and this hypothesis is reinforced, at least partially, by our results: first, two cDNAs (MsCHSI and MsCHSII) have been isolated out of several positive clones detected in the alfalfa cDNA library, and, second, several bands were detected in a Southern blot analysis (data not shown). Moreover, using oligonucleotides specifically designed from the 3′ region, we found that the MsCHSI transcript accumulation was always higher than that of MsCHSII. Taking into account that we have analyzed only two CHS genes, we cannot ascertain whether the designed oligonucleotides are specific only for these transcripts. They might hybridize with other CHS transcripts bearing in their 3′ end a sequence homologous to MsCHSI or MsCHSII.

Our results demonstrate that the response of *M. sativa* to infection with pathogenic bacteria, either incompatible or compatible, is similar in leaves and roots. As expected, the regulation of putative defense responses (as ascertained by analyses of genes involved in the synthesis of isoflavonoids or encoding PR proteins) seems to be similar to the regulation of defense genes in other plant species. The available defense gene probes now allow us to study the possible involvement of plant defense reactions during the establishment of nitrogen-fixing symbiosis with *Rhizobium meliloti*.

MATERIALS AND METHODS

Growth of plants.

M. sativa cv. Nagyszénasi was used throughout. For experiments on leaves, plants were grown in trays with vermiculite and watered with a mineral nutrient solution under controlled environmental conditions (27° C and a cycle of 16 hr of light and 8 hr of darkness). For experiments on roots, plants were grown hydroponically with the same solution and under the same environmental conditions.

Growth of bacteria.

X. campestris strains (provided by R. E. Stall, University of Florida) and *P. s.* pv. *pisi* (provided by J. Schmidt, INRA, Versailles) were grown in YDA medium (glucose, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and peptone [Difco], 5 g L^{-1}) at 30° C.

Injection of plants.

Bacteria grown overnight in YDA medium were collected by centrifugation and then resuspended in 10 mM $MgCl_2$ at a concentration of 10^9 cells per milliliter. The bacterial suspension (approximately 30 µl) was injected into leaves of 6-week-old plants with a hypodermic syringe. Control leaves were infiltrated with 10 mM $MgCl_2$. For RNA extractions, leaves were harvested 1.5 to 30 hr after infiltration and were immediately frozen in liquid nitrogen. Roots of 4-week-old plants were gently wounded with sandpaper before immersion in the bacterial suspension for 30 min. After careful washing in 10 mM $MgCl_2$, the plants were returned to an axenic nutrient solution for 1.5 to 17 hr and then were frozen in liquid nitrogen.

Monitoring bacterial growth.

The number of viable bacteria per square centimeter of leaf was determined by punching out disks of leaf tissue with a cork borer (0.7 cm in diameter) from within the inoculated area (outlined with a nontoxic marker immediately after infiltration) or from the zone adjacent to the inoculated area. The disks were ground with a mortar and pestle in 10 mM $MgCl_2$, and the number of viable bacteria was determined by plating on YDA solid medium.

Preparation of RNA.

Frozen leaves or roots were ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from leaves according to the procedure of Hall *et al.* (1978) and from roots by the guanidium–thiocyanate–cesium chloride procedure (Maniatis *et al.* 1982). The poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography and titrated with poly(U) hybridizations as previously described (Tessier and Esnault 1980; Trapy *et al.* 1981), using [^{3}H]poly(U) at 207.2 GBq mmol^{-1} (NEN).

RNA blot analysis.

Poly(A)$^+$ RNA was denatured in 50% formamide plus 6% formaldehyde at 65° C for 3 min. For Northern hybridizations, samples were fractionated on 1% agarose gel containing formaldehyde (6%) and blotted onto Hybond-N membrane (Amersham). For dot blots, a BioRad Bio-Dot apparatus was used to spot serial dilutions of denatured RNA samples onto Hybond-N membrane soaked in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After UV fixation, RNA blots were hybridized with ^{32}P-labeled cDNA inserts (using NEN Random primer plus extension labeling system and [α-^{32}P]dCTP [Amersham] at 110 TBq mmol^{-1}) or with end-labeled oligonucleotides (using T4 polynucleotide kinase and [γ-^{32}P]ATP [Amersham] at 185 TBq mmol^{-1}). Hybridizations with cDNA probes were carried out at 42° C in 50% formamide according to instructions from Amersham. Final washings were done in 0.1× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, plus 10 mM EDTA) plus 0.1% sodium dodecyl sulfate at 45° C for heterologous probes and in the same buffer at 50–60° C for homologous probes. Hybridizations with oligonucleotides were performed as described for 17-mers (Buffard *et al.* 1990).

Construction of the cDNA library.

Double-stranded cDNA was synthesized from poly(A)$^+$ RNA extracted from leaves 4 hr after infiltration with *P. s.* pv. *pisi* at 10^9 cfu ml^{-1}, by means of the Amersham cDNA Synthesis System. The cDNA was then used to prepare a phage λgt10 library (Amersham System).

Oligonucleotide screening.

Mixed oligonucleotides, synthesized by Eurogentec (Seraing, Belgium), were end-labeled with T4 polynucleotide kinase and [γ^{32}P]ATP at 111 TBq mmol^{-1} (NEN). The plaques were screened with the oligonucleotide mixtures (see Results) as a hybridization probe at 37° C by the method of Woods *et al.* (1982). Final washings were done at 50° C in 6× SSC plus 0.05% sodium pyrophosphate.

PR protein oligonucleotides.

With the aid of the Clustal (PC/Gene, Intelligenetics) and Oligo (National Biosciences) programs, two oligonucleotides (designated Oprp_1 and Oprp_2) were designed from highly conserved coding regions deduced from PR protein–encoding cDNAs isolated from pea (clones pI49 and pI176, showing an overall identity of 95%) (Fristensky *et al.* 1988) or soybean (clone p1715) (C. Sass and J. P. Jouanneau, unpublished results). These oligonucleotides (the position of the corresponding oligopeptides of which is shown in Fig. 9) were

Oprp_1 (coding strand)

5′ GT(C/G)-AC(A/C/T)-GA(C/T)-GC(A/C/T)-GA(C/T)-AA(C/T)-CT 3′
V T D A D N L

Oprp_2 (complementary strand)

3′ TG(T/G/C/A)-TT(T/C)-CC(T/A)-CT(G/A)-CG(T/G/C/A)-CG 5′

(equivalent to T K G D A A)

PCR amplification.

The cDNAs were prepared from polyadenylated RNAs extracted 6 hr after infection with *P. syringae.* In each synthesis 10 fmol of cDNAs was used with 1 mM primer, 200 μM of each deoxynucleoside triphosphate, 5 units of Taq polymerase (Gibco-BRL), and the buffer concentration recommended by this manufacturer. The reaction cycles were 94° C for 1 min, 37° C for 2 min, and 72° C for 3 min for three cycles; then 94° C for 1 min, 45° C for 2 min, and 72° C for 3 min for a total of 30 cycles; and 94° C for 1 min, 45° C for 2 min, and 72° C for 15 min for the last cycle.

Subcloning and DNA sequencing.

Inserts were isolated from DNA extracted from positive plaques and were subcloned into the *Eco*RI site of pUC19.

The PCR products were electrophoresed on agarose gels, electroeluted, blunt-ended with T4 DNA polymerase, and phosphorylated with polynucleotide kinase (Pharmacia). They were subcloned into pUC19.

Nucleotide sequences were determined by the dideoxynucleotide chain termination method (T7 sequencing kit [Pharmacia] and deoxyadenosine 5′-[α-^{35}S]thiotriphosphate at 55.5 TBq mmol^{-1} [NEN]).

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