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Differential Expression of Tomato Proteinase Inhibitor I and II Genes During Bacterial Pathogen Invasion and Wounding

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Expression of proteinase inhibitor I and II genes was investigated during infection by *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck disease in tomato. Inoculation of leaves with *P. s.* pv. *tomato* of two inbred tomato lines that are resistant and susceptible to the pathogen resulted in the accumulation of proteinase inhibitor I and II mRNAs in this organ. Our data showed that in the lines used in this study, proteinase inhibitor II mRNAs accumulated in leaves to higher levels than proteinase inhibitor I mRNA in response to *P. s.* pv. *tomato* infection and wounding. Proteinase inhibitor II mRNAs accumulated more rapidly in disease-resistant than in disease-susceptible plants. Proteinase inhibitor I mRNAs were first detected in the disease-susceptible line during infection and wounding. In contrast to wounding, the systemic induction of these genes during pathogen ingression was limited. These data show that the plant proteinase inhibitors constitute one of the components of the plant defense system that are induced in response to bacterial pathogen invasion.

Additional keywords: Lycopersicon esculentum, gene expression, plant defense.

Proteins that inhibit the activity of enzymes are widespread in the plant kingdom (Garcia-Olmedo *et al.* 1988). Many of these proteins are proteinase inhibitors directed toward inactivation of serine endopeptidases of animals and microorganisms (Richardson 1977). A few plant proteinase inhibitors affect plant proteases; they are present in low concentrations and may protect plants from the accidental release of proteases or may regulate proteolysis during development (Ryan 1973). Most plant serine proteinase inhibitors accumulate to high concentrations in plant storage organs (5–10%) and in wounded leaves (1–2%) (Ryan 1989). The significance of the high levels of these endopeptidase inhibitors is not understood, but roles in protecting plant seed reserves from premature depletion before germination, ensurance of endozotic seed dispersal, and protecting plants from chewing insects and limiting pathogen ingression have been suggested (Ryan 1973).

During mechanical wounding and in response to chewing insects, two classes of serine proteinase inhibitors accumulate at the site of physical damage and systemically in tomato plants (Green and Ryan 1972; Cleveland and Black 1982). These inhibitors are synthesized as preproteins (Nelson and Ryan 1980) and accumulate in the central vacuole (Walker-Simmons and Ryan 1977). Proteinase inhibitor I genes encode a 7,800-Da protein that contains a reactive site directed toward chymotrypsin. Proteinase inhibitor II genes encode a 12,300-Da polypeptide that harbors two reactive sites that determine specificity toward trypsin and chymotrypsin. Amino acid and nucleotide sequence data for a number of plant proteinase inhibitors have demonstrated the primary structure of these proteins. Comparisons of the tomato and potato proteinase inhibitor I and II genes have indicated a high degree of nucleotide sequence conservation. For example, potato and tomato proteinase inhibitor I genes exhibit 71% nucleotide sequence identity (Lee *et al.* 1986).

Two signals have been identified that may directly or indirectly regulate the expression of proteinase inhibitor genes. Recently, the role of abscisic acid in the systemic induction of potato and tomato proteinase inhibitor II genes was reported (Peña-Cortes *et al.* 1988). In addition, the role of the proteinase-inhibitor-inducing factor in mediating the local induction of proteinase inhibitors I and II in tomato cells is well established (Walker-Simmons and Ryan 1977). This signal is an endogenous tomato polysaccharide that is released from tomato cell walls in response to tomato or fungal polygalacturonases (Bishop *et al.* 1981). In addition, proteinase inhibitor activity can also be elicited by polysaccharides of diverse origin. For example, pectic fragments and chitosans from fungal cell walls or arthropod cuticles were also capable of inducing proteinase inhibitor activity (Walker-Simmons and Ryan 1977; Walker-Simmons *et al.* 1981). Several results suggest that proteinase inhibitors may be induced during pathogen ingression. Because plants are known to synthesize chitinases and β-glucanases that are capable of degrading bacterial and fungal cell walls in response to pathogen ingression (Boller *et al.* 1988), fragments of the pathogen's cell wall may induce the complex host response to curtail pathogen spread. Also, because in nature pathogens often gain access to plant tissues through wounds and because polysaccharide fragments are released during wounding events, host defense mechanisms may be active preceding pathogen invasion. This would accelerate a plant's ability to restrict

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the development of disease. This theory gains further support given the fact that many other genes that encode enzymes important in a plant's defense strategy are also induced by polysaccharide fragments. Most of these gene products accumulate in the region immediately surrounding the site of pathogen invasion. These proteins include β-glucanases and chitinases that degrade fungal and bacterial cell walls (Hedrick *et al.* 1988), enzymes for phytoalexin and lignin biosynthesis (Bell *et al.* 1986; Mehdy and Lamb 1987; Walter *et al.* 1988) and structural proteins, such as hydroxyproline-rich glycoproteins (Showalter *et al.* 1985).

Although considerable insights have been gained in the evolution (Lee *et al.* 1986) and expression of proteinase inhibitor genes in response to wounding (Sanchez-Serrano *et al.* 1987; Thornburg *et al.* 1987; Peña-Cortes *et al.* 1988), little is known about the expression of these genes in response to bacterial pathogen invasion. We have monitored changes in proteinase inhibitor I and II gene expression in tomato inbred lines that are resistant and susceptible to the causal agent of bacterial speck disease, *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al.* Differences in the temporal and systemic accumulation of proteinase inhibitor I and II RNAs during compatible and incompatible interactions with *P. s.* pv. *tomato* were documented. In addition, the levels of proteinase inhibitor I and II mRNAs in the resistant and susceptible lines were significantly different in response to wounding.

MATERIALS AND METHODS

Plant material—infection and wounding. Inbred cultivars of *Lycopersicon esculentum* Mill. that exhibit resistance (incompatible interaction) or susceptibility (compatible interaction) to the causal agent of bacterial speck, *P. s.* pv. *tomato*, were kindly provided by J. Williams (Petoseed Company, Saticoy, CA). The line Peto 238R is homozygous for the dominant resistance gene, *Pto*-1. The *Pto*-1 gene was originally derived from the tomato cultivar W1170 (Pitblado and Kerr 1980). Peto 238S is susceptible to *P. s.* pv. *tomato* and does not harbor the *Pto*-1 resistance gene. Healthy, wounded, and infected plants were grown in the greenhouse under natural daylight cycles. Populations of plants were split for infection and wounding treatments.

P. s. pv. *tomato*, strain PT11, was provided by D. Cooksey (University of California, Riverside, CA). *P. s.* pv. *tomato* was grown for 48 hr at 18° C on King's B agar medium and harvested into sterile water with a sterile spatula, and the concentration of the bacterial suspension was adjusted to 3×10^8 bacteria per milliliter ($OD_{590\ nm} = 0.4$). Greenhouse-grown tomato plants at the three- to four-leaf stage were used in these studies unless otherwise indicated. To eliminate plant-to-plant variation as a source of error in all experiments described, six to eight tomato plants were used for each experimental time point; leaves from these plants were pooled at the time of harvesting. Leaves were gently inoculated with the bacterial suspension with cotton swabs. Bacterial speck lesions were visible on Peto 238S leaves within 72 hr; no visible symptoms were detected on Peto 238R leaves 5 days postinoculation. Infected disease-resistant and -susceptible tomato leaves were harvested 24 hr after inoculation. Mock-infected tomato leaves were gently swabbed with sterile distilled water and harvested 24 hr later. Leaves were harvested directly into liquid nitrogen and stored at −80° C. The infection time course was conducted as described above and infected leaves were harvested at 3, 6, 9, 12, 24, 36, 48, and 72 hr after inoculation. To investigate systemic induction of proteinase inhibitor genes in response to *P. s.* pv. *tomato* invasion, plants at the five- to six-leaf stage were used. The third and fourth leaves (middle leaves) from the tomato plant apex were inoculated with *P. s.* pv. *tomato* as described above. At 24, 36, 48, and 72 hr after inoculation, middle infected leaves and uninoculated upper and lower leaves were harvested and stored as described above.

Tomato leaves were wounded by crushing leaflets with pliers. Several wounds were made perpendicular to the main vein. During the wounding time-course experiments, leaves were wounded and harvested 3, 6, 9, 12, and 24 hr later. To investigate systemic induction of the proteinase inhibitor genes in response to wounding, plants at the five- to six-leaf stage were used. The third and fourth leaves (middle leaves) from the tomato apex were wounded. At 6, 12, and 24 hr after wounding, wounded middle leaves and nonwounded upper and lower leaves were harvested as described above. Overwounded leaves were wounded twice before harvest. Leaves were wounded as described above and 18 hr later were rewounded; leaves were harvested 6 hr later. Multiple wounding events distinguish the overwounding regimen from that used in the systemic and temporal time course experiments.

Nucleic acid isolation. Polysomal poly(A)$^+$ RNA was isolated from control, mock-infected, overwounded, and infected leaves as previously described (Chang and Walling 1991). For infection time course experiments, total poly(A)$^+$ RNA was isolated according to Collins and Hague (1983) with the following modification: RNAs were pelleted through an 8-ml CsCl cushion and centrifuged in a Beckman 60Ti rotor (Beckman Instruments, Fullerton, CA) for 19 hr at 30,000 rpm (90,600 × *g*). RNA pellets were washed in 70% ethanol and subsequently with 7 M urea/2% sodium dodecyl sulfate. Poly(A)$^+$ RNA was isolated as described by Rosek *et al.* (1978). Total RNAs from wounded leaves were isolated according to Chirgwin and Glisin (Glisin *et al.* 1974; Chirgwin *et al.* 1979). Proteinase inhibitor I and II in clones, pT1-24 and pT2-47, have been described (Graham *et al.* 1985a, 1985b) and were kindly supplied by Clarence Ryan (Washington State University, Pullman, Washington). Plasmid DNAs were prepared according to Clewell and Helinski (1969).

RNA blot hybridizations. RNA was size fractionated on 1.5% formaldehyde gels as described by Lehrach *et al.* (1977), blotted and UV-cross-linked to Hybond-N filters (Amersham, Arlington Heights, IL; Khandjian 1986). RNA blots were hybridized and washed as described by Saito *et al.* (1989). ^{32}P-labeled pT1-24 and pT2-47 probes (specific activity = 1×10^9 cpm/μg) were synthesized by using random oligonucleotide primers and [^{32}P]dATP (New England Nuclear, Boston, MA)) as described by Feinberg and Vogelstein (1983). Filters were exposed to Kodak XAR5 film for 2–20 hr with one Quanta III intensifying screen (DuPont) at −80° C. Autoradiographic signals were

quantitated by scanning autoradiograms in a linear exposure range with an LKB Ultra Scan XL (Pharmacia LKB Biotechnology AB, Bromma, Sweden) laser densitometer. BRL RNA markers (0.24- to 9.5-kb RNA ladder; Bethesda Research Laboratories, Gaithersburg, MD) were electrophoresed in parallel lanes to determine mRNA sizes. To support densitometric quantitation data, proteinase inhibitor I and II RNAs were quantitated by using RNA dot blot hybridizations. mRNAs were denatured and immobilized on Hybond-N filters as described by Chang and Walling (unpublished results). RNA dot blots were hybridized and washed as described by Saito *et al.* (1989). RNA dots were localized by autoradiography, excised, and counted by liquid scintillation spectrophotometry (Beckman LS1700). RNA blot and dot blot studies were repeated at least twice. Hybridization of RNA blots with a tomato ribulose-1,3-bisphosphate carboxylase small subunit gene probe indicated that RNAs loaded per lane were equal.

RESULTS

Accumulation of proteinase inhibitor I and II mRNAs during infection and in response to wounding. To evaluate the accumulation of proteinase inhibitor I and II mRNAs during *P. s.* pv. *tomato* infection and during mechanical wounding, polysomal poly(A)$^+$ mRNAs were isolated from healthy leaves, overwounded leaves, and leaves harvested 24 hr after inoculation with *P. s.* pv. *tomato*. The level of proteinase inhibitor I and II mRNAs from the disease-resistant and disease-susceptible lines were compared. Proteinase inhibitor I and II and their mRNAs are known to accumulate rapidly and to high levels in tomato leaves in response to mechanical wounding and herbivorous pests (Rhodes 1979; Graham *et al.* 1986). Proteinase inhibitor I mRNA was not detected on healthy tomato leaf polysomes (Fig. 1) or in mock-infected leaves (data not shown). Proteinase inhibitor I mRNA accumulated to high levels in overwounded leaves in both disease-susceptible and disease-resistant leaves. Proteinase inhibitor I mRNA was barely

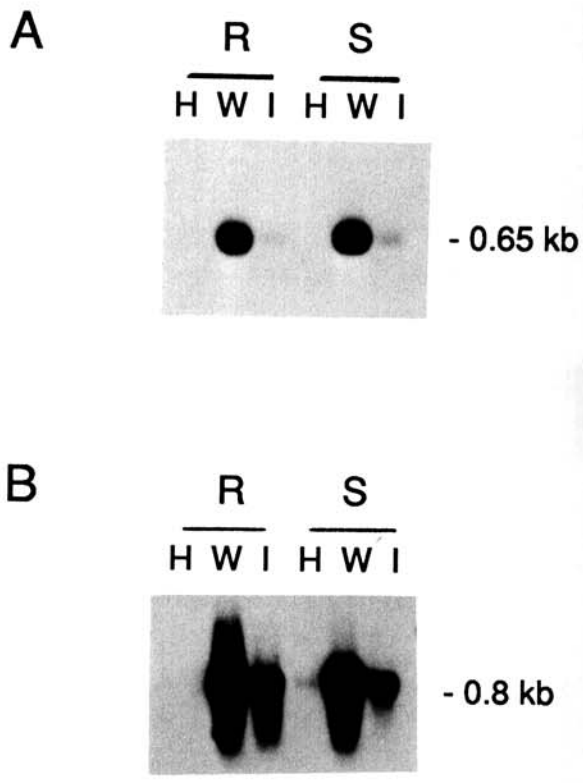

Fig. 1. Accumulation of proteinase inhibitor I and II mRNAs in healthy, wounded, and infected leaves. Two micrograms of poly(A)$^+$ polysomal mRNA from healthy (H), wounded (W), and 24-hr infected (I) leaves were fractionated on a 1.5% formaldehyde gel and UV-cross-linked to nylon filters. RNA blots were hybridized with **A,** a ^{32}P-labeled proteinase inhibitor I clone, pT1-24 or, **B,** proteinase inhibitor II clone, pT2-47. The size of the proteinase inhibitor I and II mRNAs are indicated in kilobases (kb). Autoradiograms were exposed for 16 (A) and 18 hr (B) with one screen at −80° C.

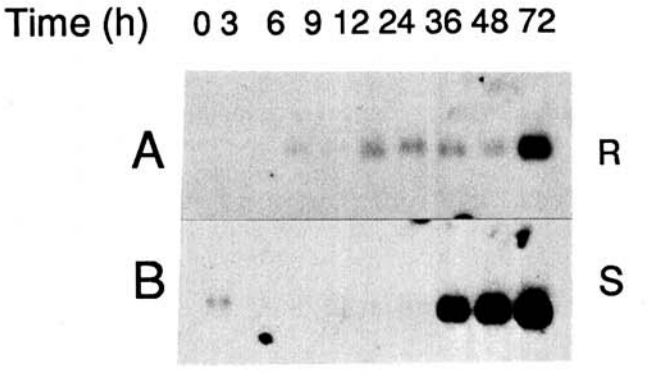

Fig. 2. Accumulation of proteinase inhibitor I mRNA in disease-resistant and -susceptible plants after *Pseudomonas syringae* pv. *tomato* inoculation. Two micrograms of poly(A)$^+$ RNA was isolated from tomato-infected leaves 3, 6, 9, 12, 24, 36, 48, and 72 hr after inoculation with *P. s.* pv. *tomato*, were fractionated on formaldehyde gels, and UV-cross-linked to nylon filters. RNA blots were hybridized with a ^{32}P-labeled probe pT1-24 probe. **A,** mRNAs from the disease-resistant plants. **B,** mRNAs from the disease-susceptible plants. Autoradiograms were exposed for 18 hr at −80° C with one screen.

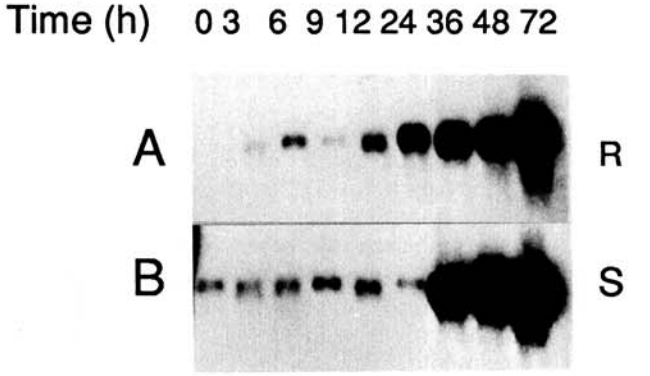

Fig. 3. Accumulation of proteinase inhibitor II mRNA in disease-resistant and -susceptible plants after *Pseudomonas syringae* pv. *tomato* inoculation. Two micrograms of poly(A)$^+$ RNA was isolated from control and infected leaves 3, 6, 9, 12, 24, 36, 48, and 72 hr after inoculation were fractionated on 1.5% formaldehyde gels, and UV-cross-linked to nylon filters. RNA blots were hybridized with a ^{32}P-labeled pT2-47 probe. **A,** mRNAs from the disease-resistant plants. **B,** mRNAs from the disease-sensitive plants. Autoradiograms were exposed for 15 hr at −80° C with one screen.

detected 24 hr after inoculation with *P. s.* pv. *tomato* in both disease-resistant and -susceptible lines (Fig. 1A).

To assess the relative abundance of proteinase inhibitor II mRNA that accumulated in healthy, overwounded, and *P. s.* pv. *tomato*-infected leaves, RNA blots (Fig. 1B) were hybridized to a proteinase inhibitor II probe, pT2-47. These data indicated that similar to proteinase inhibitor I mRNA, the proteinase inhibitor II mRNA was not detected on polysomes of healthy leaves and was induced to high levels after wounding in both disease-resistant and -susceptible tomato leaves. This mRNA was not detected in mock-infected leaves (data not shown). Proteinase inhibitor II mRNA was also induced during *P. s.* pv. *tomato* invasion, although its level of expression was distinct from that observed for proteinase inhibitor I mRNA. By 24 hr after *P. s.* pv. *tomato* infection, proteinase inhibitor II mRNA was detected in both disease-resistant and -susceptible leaves.

Temporal accumulation of proteinase inhibitor I and II in infected leaves. The temporal expression of proteinase inhibitor I genes in response to bacterial pathogen invasion was determined by RNA blot hybridization experiments. Poly(A)$^+$ RNAs were isolated from disease-resistant and -susceptible leaves at various times after inoculation with *P. s.* pv. *tomato*. Differential accumulation of proteinase inhibitor I mRNA was observed in the disease-resistant and -susceptible lines (Fig. 2). During the incompatible interaction, proteinase inhibitor I RNA was barely detectable until 12 hr postinoculation. Between 48 and 72 hr after infection, proteinase inhibitor I mRNA levels increased dramatically. The accumulation of proteinase inhibitor I RNA in disease-susceptible plants was delayed relative to the incompatible interaction. In disease-susceptible plants, proteinase inhibitor I mRNA was barely detected until 24 hr postinoculation. By 36 hr of infection, proteinase inhibitor I RNA accumulated to high levels, which continued to increase over the next 36 hr. Quantitation of proteinase inhibitor I RNA by RNA dot blot hybridization studies showed that the maximal accumulation of proteinase inhibitor I mRNAs during the compatible interaction was 2.7-fold greater than that attained during the incompatible interaction.

The accumulation of proteinase inhibitor II mRNA during *P. s.* pv. *tomato* infection was distinct from that observed for the proteinase inhibitor I mRNA. Proteinase inhibitor II mRNA was readily detected during compatible and incompatible interactions by 12 hr postinoculation. In disease-resistant leaves, proteinase inhibitor II RNA began to accumulate to higher levels between 12 and 24 hr after infection and mRNAs increased progressively to maximal levels by 72 hr postinoculation (Fig. 3A). In contrast, there was a rapid increase in proteinase inhibitor II RNA between 24 and 36 hr after inoculation in disease-susceptible leaves (Fig. 3B). In addition, the maximal level of proteinase inhibitor II mRNA induced during the compatible interaction was 2.8-fold greater than that observed during the incompatible interaction.

Systemic accumulation of proteinase inhibitor I and II RNAs in response to *P. s.* pv. *tomato*. Because proteinase inhibitor mRNAs and enzymes are systemically induced in tomato and potato leaves in response to mechanical wounding, polysaccharide application, and abscisic acid (Bishop *et al.* 1981; Peña-Cortes *et al.* 1988; Peña-Cortes *et al.* 1989), it was important to determine if they were systemically induced during *P. s.* pv. *tomato* invasion. Total RNAs were isolated from infected middle leaves and noninfected upper and lower leaves. These RNAs were subjected to RNA blot hybridization studies. Proteinase inhibitor I RNA was induced in infected middle leaves at low levels by 36 hr postinoculation in the disease-resistant line (Fig. 4A). The mRNA dramatically increased in middle leaves by 48 hr and decreased by 72 hr after infection. In the disease-susceptible line, proteinase inhibitor I RNA accumulated to high levels by 36 hr and reached maximal levels by 48 hr after infection. Like the incompatible interaction, proteinase inhibitor I RNA decreased by 72 hr in the compatible interaction.

In this experiment the time of appearance and the time that maximal levels of proteinase inhibitor RNA were observed in inoculated leaves during the incompatible interaction were slightly accelerated relative to infection time course displayed in Figure 2. The difference in the timing of proteinase inhibitor gene expression during the two infection cycles may reflect the different ages of the tomato plants used in the two studies (see Materials and Methods) or slight variability in the greenhouse environment during the two growth cycles. Similar variability was previously observed in wounding experiments in tomato seedlings (Graham *et al.* 1986). It is important to note that despite the slight temporal shift, the overall pattern of proteinase inhibitor gene expression in Figures 2 and 4 is retained in the time-course experiments. Proteinase inhibitor I mRNAs were more prevalent in the disease-susceptible plant leaves than in disease-resistant plant leaves during the infection period.

Systemic induction of proteinase inhibitor I RNA during *P. s.* pv. *tomato* infection was observed (Fig. 4). Proteinase inhibitor I RNA was not detected in lower noninfected leaves during compatible or incompatible interactions. During compatible and incompatible interactions, proteinase inhibitor I RNAs were more abundant in the infected leaves than in the noninfected upper leaves. The temporal accumulation of proteinase inhibitor I RNA in noninfected upper leaves paralleled its accumulation in middle infected leaves. For example, in both disease-resistant and -susceptible plants, maximal levels of proteinase inhibitor I RNA were detected 48 hr after infection and subsequently decreased by 72 hr. Despite their similarity in timing, levels of proteinase inhibitor I RNAs in noninfected upper leaves differed quantitatively when the disease-resistant and -susceptible lines were compared; higher levels were detected in the disease-resistant line.

The systemic induction of proteinase inhibitor II RNAs was similar to that observed for proteinase inhibitor I RNAs (data not shown). Two minor variations were noted. First, proteinase inhibitor II RNAs were first detected in the middle infected and noninfected upper leaves by 24 hr after infection. Second, the decline of proteinase inhibitor II RNAs by 72 hr was greater in disease-resistant than -susceptible leaves.

Temporal accumulation of proteinase inhibitor I and II RNAs during mechanical wounding. The temporal accumu-

lation of proteinase inhibitors I and II mRNAs indicated that proteinase inhibitor II mRNA accumulated more rapidly than proteinase inhibitor I mRNA during compatible and incompatible *P. s.* pv. *tomato*/tomato interactions (Figs. 2–4). These results were intriguing because proteinase inhibitor I mRNAs were more rapidly induced in the cultivar Bonnie Best during wounding than proteinase inhibitor II mRNAs (Graham *et al.* 1986). These results suggested that either proteinase inhibitors I and II accumulated differently in response to wounding and *P. s.* pv. *tomato* infection or that the regulation of proteinase inhibitor genes in the cultivar Bonnie Best and the inbred disease-resistant and -susceptible lines were distinct. To discriminate between these alternatives, the accumulation of proteinase inhibitor I and II RNAs after mechanical wounding was evaluated by RNA blot hybridizations. In disease-susceptible leaves, proteinase inhibitor I mRNAs accumulated more rapidly than in disease-resistant leaves (Fig. 5A,B). As early as 3 hr after wounding, proteinase inhibitor I mRNA was detected in disease-susceptible leaves. This level rose dramatically by 6 hr and then gradually increased to reach maximal levels by 24 hr postwounding. The induction of proteinase inhibitor I RNA in the disease-resistant line was observed by 6 hr after wounding. This RNA reached maximal levels by 9 hr, which were maintained for the next 15 hr.

Proteinase inhibitor II RNA accumulated more rapidly than proteinase inhibitor I RNA in the disease-resistant and -susceptible lines in response to wounding. Three hours after wounding, proteinase inhibitor II RNA was clearly detected in both lines (Fig. 5C,D). In disease-resistant tomato leaves, proteinase inhibitor II RNA dramatically increased by 6 hr and continued to increase over the next 6 hr after wounding. A small decrease in proteinase inhibitor II RNA was observed 24 hr after wounding. In disease-susceptible tomato leaves, the accumulation of proteinase inhibitor II RNA was delayed relative to the disease-resistant line; a large increase of proteinase inhibitor II RNA occurred between 6 and 9 hr after wounding. Quantitative RNA dot blot studies indicated that proteinase inhibitor II accumulated to 2.4- to 2.9-fold higher levels than proteinase inhibitor I during wounding (data not

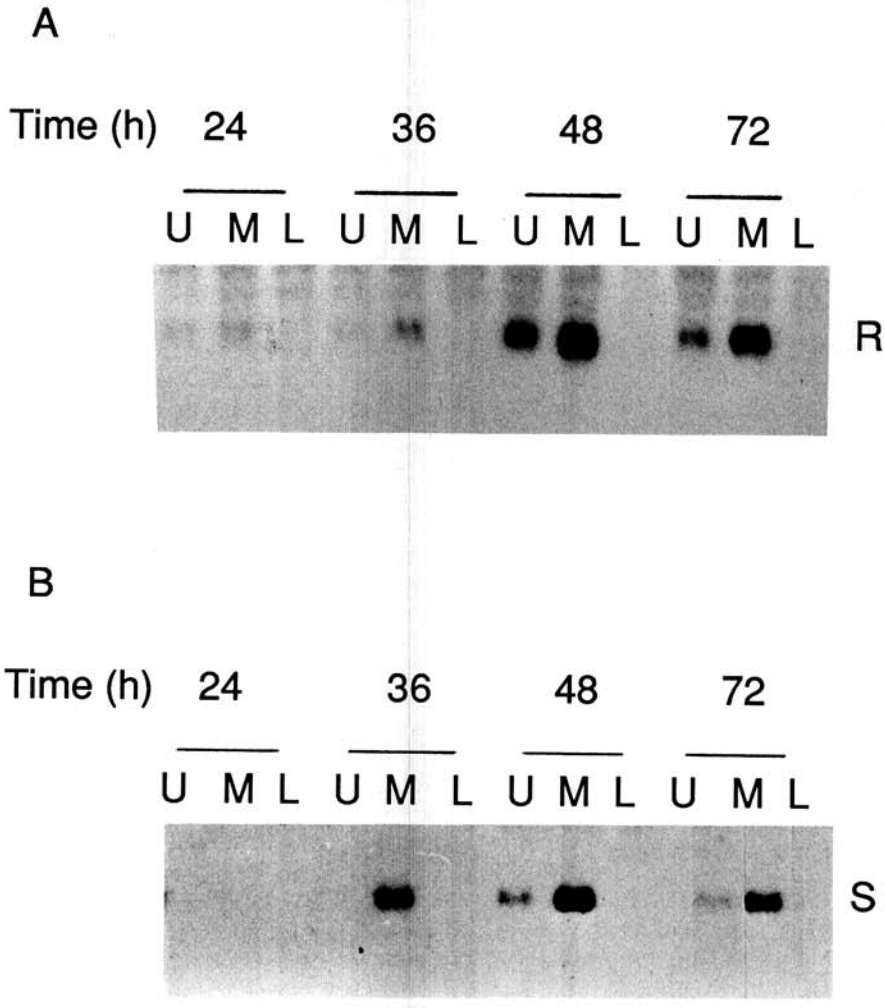

Fig. 4. Temporal and systemic induction of proteinase inhibitor I RNA in response to *Pseudomonas syringae* pv. *tomato*. **A,** Disease-resistant and, **B,** disease-susceptible plant middle tomato leaves were inoculated with *P. s. tomato* 24, 36, 48, and 72 hr after infection; upper (U), middle (M), and lower (L) leaves were harvested. Fifteen micrograms of total RNA was fractionated on 1.5% formaldehyde gel and UV-cross-linked to nylon filters. RNA blots were hybridized to a ^{32}P-labeled pT1-24 probe. Autoradiograms were exposed for 20 hr at −80° C with one screen.

shown).

Systemic accumulation of proteinase inhibitor I and II mRNAs during wounding. The systemic induction of proteinase inhibitor I and II RNAs by wounding in the disease-resistant and -susceptible lines was examined by RNA blot analysis. Similar to the response to bacterial pathogen invasion, during wounding proteinase inhibitors I and II RNAs accumulated in wounded middle leaves and in nonwounded upper leaves (Fig. 6). These RNAs were not detected in nonwounded lower leaves. Unlike the response to bacterial pathogen infection, the proteinase inhibitor I and II RNAs accumulated to levels equal to or greater than that detected in the middle wounded leaves. For example, 12 hr after wounding, proteinase inhibitor I RNAs in upper leaves exceeded the levels detected in the middle leaves. (Fig. 6A,B). In contrast, proteinase inhibitor II RNAs were present at similar levels in both wounded and nonwounded upper leaves (Fig. 6C,D). By 24 hr, the proteinase inhibitor I and II RNAs decreased in all samples.

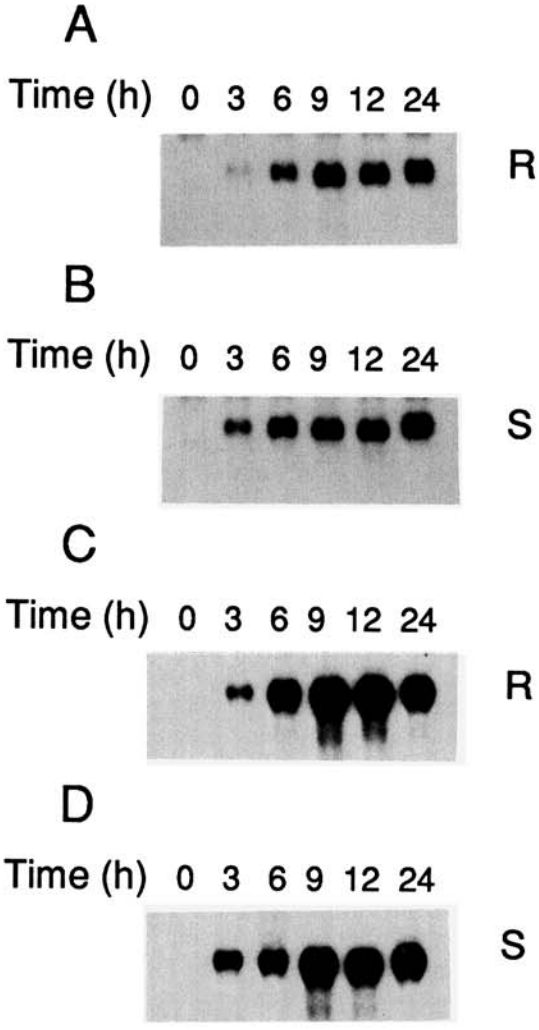

Fig. 5. Induction of proteinase inhibitor I and II mRNAs after wounding in disease-resistant and -susceptible plants. **A, C,** Disease-resistant and, **B, D,** disease-susceptible leaves were wounded and harvested 3, 6, 9, 12, and 24 hr later. Control leaves (O) were not wounded. Fifteen micrograms of total RNA was fractionated on formaldehyde gels and UV-cross-linked to nylon filters. RNA blots were hybridized to ^{32}P-labeled pT1-24 (A and B) or pT2-47 (C and D) probes. Autoradiograms were exposed for 2 hr at −80° C with one screen.

DISCUSSION

Recent studies have significantly advanced our understanding of plant proteinase inhibitor gene expression and gene evolution. The serine endopeptidases, proteinase inhibitors I and II, accumulate in tomato and potato leaves at site of mechanical wounding and systemically throughout the plant. In addition, these proteinase inhibitors accumulate in healthy potato tubers. The accumulation of proteinase inhibitor I and II mRNAs was shown to be strictly correlated with the regional and systemic induction of proteinase inhibitor enzyme activity. Our studies have inves-

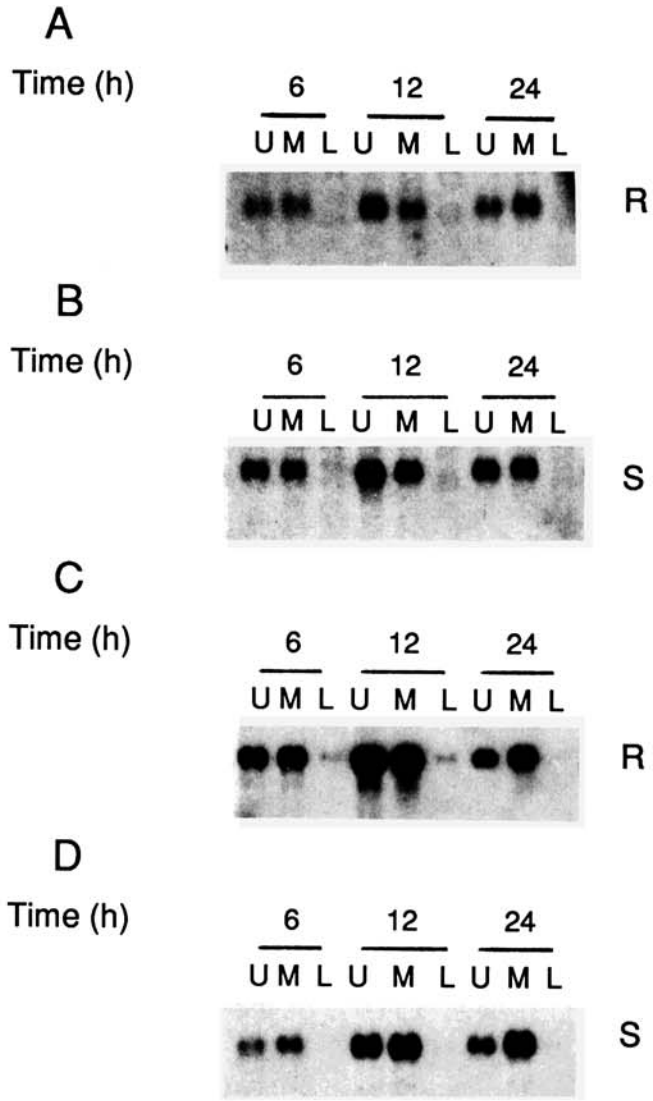

Fig. 6. Temporal and systemic induction of proteinase inhibitor I and II mRNAs in response to wounding. **A,** Disease-resistant and, **B,** disease-susceptible middle tomato leaves were wounded. Upper (U), middle (M), and lower (L) leaves were harvested 3, 6, 9, 12, and 24 hr after wounding. Fifteen micrograms of total RNA was fractionated on 1.5% formaldehyde gels and UV-cross-linked to nylon filters. RNA blots were hybridized to a ^{32}P-labeled pT1-24 (A and B) or pT2-47 (C and D) probes. Autoradiograms were exposed for 3 hr at −80° C with one screen.

tigated the accumulation of proteinase inhibitor I and II mRNAs in response to wounding and during bacterial pathogen invasion of inbred tomato lines. Our data showed that in the disease-resistant and -susceptible lines, proteinase inhibitor II RNA accumulated to higher levels than proteinase inhibitor I mRNA in response to wounding and during *P. s.* pv. *tomato* infection. The RNA blot data presented here and quantitative RNA dot blot experiments (data not shown) have indicated that in both inbred tomato lines, proteinase inhibitor II in RNA accumulated to higher levels than proteinase inhibitor I in response to wounding and *P. s.* pv. *tomato* infection (data not shown). Previous studies with the tomato cultivar Bonnie Best have shown that in response to mechanical wounding, proteinase inhibitor I mRNAs accumulated more rapidly than proteinase inhibitor II mRNAs in leaves (Graham *et al.* 1986). The differences between our data and previous observations with the cultivar Bonnie Best are likely to reflect genotypic differences between the tomato lines used in the two studies. The observed phenotypic variation is not reflective of large changes in the structure and organization of proteinase inhibitor I and II genes as evidenced by DNA blot analysis of the disease-resistant, disease-sensitive, and Bonnie Best genomic DNAs (V. Pautot and L. L. Walling, unpublished results). Therefore, it is likely that more subtle genotypic differences alter either transcriptional or posttranscriptional events that modulate the time of appearance and abundance of proteinase inhibitor I and II RNAs in response to wounding and bacterial pathogen invasion.

As observed with the cultivar Bonnie Best, systemic induction of proteinase inhibitor I and II RNAs was documented after wounding in the disease-resistant and -susceptible cultivars. During wounding, proteinase inhibitor I and II RNAs accumulated in upper nonwounded leaves but were not detectable in nonwounded lower leaves. The level of proteinase inhibitor RNAs in the upper leaves and wounded middle leaves was similar. In contrast to the wounding response, during *P. s.* pv. *tomato* infection the systemic induction of proteinase inhibitor I and II gene expression was more limited. The levels of proteinase inhibitor I and II RNAs in the noninfected upper leaves were substantially lower that observed in infected middle leaves.

Our results indicated that during the first 72 hr of *P. s.* pv. *tomato* infection, proteinase inhibitor I and II mRNAs accumulated differentially. Quantitative RNA dot blot experiments indicated that proteinase inhibitor II RNA accumulated to 8.8- to 8.9-fold higher levels than proteinase inhibitor I mRNA during infection. Proteinase inhibitor II mRNA accumulated more rapidly in disease-resistant than in -susceptible plants. This accelerated expression of proteinase inhibitor II during the incompatible interaction is consistent with the changes in gene expression observed during compatible and incompatible fungal infections of bean. Chitinase, enzymes essential for phytoalexin and lignin biosynthesis, and hydroxyproline-rich glycoprotein RNAs accumulate more rapidly in response to an incompatible race of *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi and Cav. when compared with a compatible race (Corbin *et al.* 1987; Mehdy and Lamb 1987; Bell *et al.* 1986; Hedrick *et al.* 1988; Walter *et al.* 1988).

Another similarity observed between the *C. lindemuthianum*/*Phaseolus vulgaris* L. expression studies and our studies with *P. s.* pv. *tomato* was that the levels of mRNAs accumulated during the compatible interaction often exceeded that observed in the incompatible interaction (Bell *et al.* 1986). For example, by 72 hr, the level of proteinase inhibitor II mRNAs in disease-susceptible plants (compatible) was threefold greater than that observed in disease-resistant plants (incompatible). These results are consistent with the ingression and multiplication of *P. s.* pv. *tomato* within the inoculated leaves in the disease-susceptible line for longer periods of time than the disease-resistant line before the curtailment of the bacterial infection (Bashan *et al.* 1981). This temporal delay may allow for more tomato cells to respond to *P. s.* pv. *tomato* and express genes important in controlling disease development and, therefore, the level of proteinase inhibitor II RNA accumulated is greater.

Unlike the proteinase inhibitor II genes in this study and the chitinase, phytoalexin, and lignin biosynthesis or hydroxyproline-rich glycoprotein genes in *Phaseolus*, which are expressed more rapidly during an incompatible interaction, proteinase inhibitor I mRNAs accumulated more rapidly during the compatible interaction. These data suggest that the factors that modulate proteinase inhibitor I and II gene expression are subtly different. It is possible that their responses to the signals proteinase-inhibitor-inducing factor and abscisic acid may vary. Alternatively, unidentified factors that modulate proteinase inhibitor gene expression may be involved.

The response of plants to wounding and pathogen invasion is complex; many of the same chemicals and enzymes are induced in these two processes. Due to the wide array of biochemicals that are synthesized, it is unclear which have an important role in limiting the multiplication of a particular pathogen. For example, we demonstrated the induction of proteinase inhibitor I and II genes in response to *P. s.* pv. *tomato* infection. In addition, it has been shown that a tobacco proteinase inhibitor is induced during the hypersensitive response to tobacco mosaic virus and is a potent inhibitor of serine proteases of microbial origin (Geoffroy *et al.* 1990). Despite these data, the role of proteinase inhibitors in actively curtailing fungal, bacterial, and viral disease development has not been established. Proteinase inhibitor II has been demonstrated to be a powerful and effective mechanism for the control of insect predation (Hilder *et al.* 1987; Johnson *et al.* 1989). The fact that proteinase inhibitor I does not render transgenic tobacco plants resistant to plant predators suggests that the specificity of the proteolytic enzymes important in the pathogen-plant interaction may be an important factor in the effectiveness of proteinase inhibitors in controlling disease development or insect damage.

Several lines of evidence suggest that tomato proteinase inhibitors may be one factor important in limiting *P. s.* pv. *tomato* infection. Bashan *et al.* (1986) demonstrated that the protease activity increased in response to *P. s.* pv. *tomato* infection. Proteolytic activities reached their maximal levels by 48 hr after infection in disease-resistant plants and subsequently decreased. In disease-sensitive plants, protease activity continued to increase until 120

hr after inoculation. They observed a strong correlation between severity of disease symptoms and level of protease activity, suggesting that proteases play an important role in the development of bacterial speck disease. Although the nature of the proteases produced during *P. s.* pv. *tomato* infection has not been established, the fact that many bacterial proteases are serine proteases and the decline in protease activity correlates with the temporal induction of proteinase inhibitor I and II gene mRNAs (Figs. 2 and 3) suggests that proteinase inhibitors may be an important mechanism to curtail *P. s.* pv. *tomato* spread.

The implication of proteases in the development of bacterial speck disease contrasts to the conclusions of Tang *et al.* (1987). These investigators demonstrated that when *Xanthomonas campestris* (Pammel) Dowson strains that harbor active or inactive protease genes are infiltrated into turnip leaves, little effect on the hypersensitive response was demonstrated. It is possible that when plant tissues encounter the high bacteria concentrations that accompany infiltration, the plant's response to *Xanthomonas* and the resulting disease symptomatology are independent of the activity of bacterial-coded proteases. These experiments do not disallow a more subtle role for bacterial-encoded proteases in disease development (Daniels *et al.* 1984). The impact of bacterial proteases might only be observed when the portal for pathogen entry and the bacterial concentration in plant tissues is more analogous to that which occurs in nature. Under these conditions, it is possible that the degradation of proteins associated with the plant cell wall and plasma membrane by bacterial proteases may unlink signaling pathways essential for the induction of genes important in controlling the progression of disease. The direct role of proteinase inhibitors curtailing pathogen ingression is currently being investigated.

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