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cDNA Cloning, Structure, and Expression of a Novel Pathogenesis-Related Protein in Bean

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We have isolated cDNA clones spanning the full length of a transcript (PvPR3) encoding a novel pathogenesis-related (PR) protein in bean (*Phaseolus vulgaris*). The PvPR3 transcript accumulates gradually over 24 hr in elicitor-treated cell suspensions. This pattern of expression is distinct from those of previously reported elicitor-induced transcripts in bean. Specifically, transcripts encoding two recently described acidic bean PR proteins, phenylpropanoid pathway enzymes, accumulate to maximal levels by 4-8 hr, while hydroxyproline-rich glycoprotein mRNA accumulation is delayed by several hours. The PvPR3 mRNA also accumulates after wounding of hypocotyls with kinetics comparable to those of mRNAs encoding phenylpropanoid pathway mRNAs. PvPR3 appears to exist as a single gene within a family of approximately 15 related genes in the bean genome. The PvPR3 protein deduced from the cDNA sequences (14,950 Da, pI = 10.0) lacks a putative signal peptide suggesting a cytosolic localization. Amino acid sequence comparisons with databases revealed that PvPR3 represents a new class of PR proteins without significant sequence homology to previously characterized PR proteins or other proteins.

Additional keyword: fungal elicitor.

The metabolic response of plants to pathogen attack or wounding is characterized by the rapid and specific accumulation of numerous polypeptides as determined by *in vivo* labeling of proteins or *in vitro* translation of RNA (Cramer *et al.* 1985a; Somssich *et al.* 1986; Hadwiger and Wagoner 1983; Dalkin *et al.* 1990). Transcripts that accumulate during the defense response include those encoding enzymes involved in the synthesis of antimicrobial phytoalexins and lignin, cell wall proteins such as hydroxyproline-rich glycoproteins, and proteinase inhibitors. Many of these changes in mRNA levels have been shown to involve activation of gene transcription (for review, see Dixon and Lamb 1990).

In addition, a heterogeneous class of proteins termed pathogenesis-related (PR) proteins are found in a wide variety of infected plants. PR proteins are synthesized by plants in response to viral infection, microbial attack, and mechanical wounding (for reviews, see Van Loon 1985; Rigden and Coutts 1988; Bol *et al.* 1990; Carr and Klessig 1990). These proteins were initially defined as polypeptides with relatively low molecular weights that accumulate extracellularly in infected tissue and that show extreme isoelectric points and resistance to proteolytic degradation (Van Loon 1985). However, the properties of many designated PR proteins do not conform closely to these criteria. The well characterized tobacco PR1 proteins, both acidic and basic isoforms, are representative of one class of PR proteins of unknown function (Cornelissen *et al.* 1987). A second class of PR proteins of unknown function includes potato pSTH2 (Matton and Brisson 1989), parsley PR1 (Somssich *et al.* 1988), pea p149 (Fristensky *et al.* 1988), and bean PvPR1 and PvPR2 (Walter *et al.* 1990). A third class of PR proteins was originally characterized in tobacco and found to encode thaumatinlike proteins (Pierpoint *et al.* 1987). Two additional classes of PR proteins with known enzymatic activities are chitinases and β-1,3-glucanases (Hooft van Huijsduijnen *et al.* 1987; Legrand *et al.* 1987; Kauffmann *et al.* 1987; Kombrink *et al.* 1988).

In this paper, we describe the structure and expression of a novel PR protein-encoding gene from *Phaseolus vulgaris* L. (Pv) designated PvPR3. Overlapping cDNA clones that span the full length of the PvPR3 transcript were isolated from a cDNA library constructed from elicitor-treated bean cell suspension cultures. The elicitor used in these studies was a cell wall fraction from the fungal pathogen, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. Elicitor treatment of bean cell suspensions resulted in a gradual accumulation of the mRNA over many hours. In contrast, wounding of hypocotyls resulted in maximal accumulation of the PvPR3 transcript within a few hours. These patterns of expression are compared with those of previously described PR protein genes (PvPR1 and PvPR2) as well as other defense-activated genes that have been studied in the bean system (Walter *et al.* 1990; Bell *et al.* 1986; Mehdy and Lamb 1987; Showalter *et al.* 1985). In addition, we found that the PvPR3 protein and cDNA sequences are unique in comparison to other sequences within protein and nucleic acid databases, respectively.

MATERIALS AND METHODS

Plant cell culture and treatment with elicitor. Cells of bean (*Phaseolus vulgaris* L. 'Immuna') were grown as previously described (Dixon and Bendall 1978) except that suspensions were cultured in total darkness. *C. lindemuthianum* was grown in liquid culture as previously described (Bailey and Deverall 1971). Elicitor was prepared using the procedure of Anderson-Prouty and Albersheim (1975).

Elicitor (60 μg of glucose equivalents per milliliter) was applied to 7- to 10-day-old cell cultures, and the cells were harvested as previously described (Cramer *et al.* 1985b).

Growth and treatments of plants. For wounding studies, bean (cv. Tendergreen) seeds were germinated and grown in darkness as previously described (Shields *et al.* 1982). After 8 days, 10-mm segments were excised from the hypocotyls and incubated at 25° C in darkness in 100-mm plastic petri dishes (≈30 segments per dish) containing 10 ml of 50 μg/ml of ampicillin solution. The petri dishes were rotated at 50 rpm for aeration. Wounded plant material was harvested at various times and frozen in liquid nitrogen.

Isolation of RNA and DNA. Total RNA was isolated by either the phenol:sodium dodecyl sulfate (SDS) or guanidinium thiocyanate method as previously described (Mehdy and Lamb 1987). Genomic DNA was purified from leaves (cultivar Tendergreen) by cetyltrimethyl-ammonium bromide extraction and CsCl density gradient centrifugation as described (Murray and Thompson 1980). The bean cDNA clones H1 and Hyp3.6 have been described (Lawton and Lamb 1987; Corbin *et al.* 1987) and were kindly supplied by C. J. Lamb (Salk Institute, La Jolla, CA).

Isolation of PvPR3 cDNA clones. The construction of a cDNA library in λgtll from bean cells treated with elicitor for 3.5 hr has been described (Mehdy and Lamb 1987). Approximately 10^5 recombinants were screened at low stringency (30% formamide, 6× SSC [1× SSC = 0.15 M sodium chloride, 0.015 sodium citrate, pH 7.0], 3× Denhardt's solution, 50 mM sodium phosphate, 5 mM EDTA, 0.1% SDS, 100 μg/ml of carrier DNA at 37° C) to identify sequences homologous to the insert of a rabbit cytochrome P-450 cDNA clone, P-450-4 (Okino *et al.* 1985). The hybridization stringency was defined by washing with 0.1 M NaCl, 1.0 mM $NaPO_4$, 0.1 mM EDTA, and 0.1% SDS at 37° C. The clone λ8-2-1 was selected from a total of 21 independently isolated cross-hybridizing clones. The 530-bp insert from 8-2-1 was subcloned into the pIBI24 plasmid vector (International Biotechnologies, Inc., New Haven, CT) and designated PvPR3-5′. A clone overlapping the 3′ end of the PvPR3 transcript (195–871 nucleotides) was isolated by the polymerase chain reaction (PCR) method. Using a primer spanning nucleotides 195–212 (CCGCAGCAACTTCCATTT) in the PvPR3-5′ clone in combination with a λgtll reverse primer (TTGACAC-CAGACCAACTGGTAATG), a 676-bp fragment was amplified from 8×10^6 recombinant phage of the cDNA library (Friedman *et al.* 1990). The fragment was subcloned into the pBluescript KS+ phagemid vector (Stratagene, Inc., La Jolla, CA) and designated PvPR3-3′.

RNA and DNA filter hybridization. Total RNA was size-fractionated on formaldehyde agarose gels as described previously (Mehdy and Lamb 1987) and blotted onto Duralon membranes (Stratagene, Inc., La Jolla, CA) with 10× SSC. Filters were then UV-crosslinked and prehybridized with a solution containing 5× SSPE (1× SSPE = 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5× Denhardt's solution, 0.5% SDS, and 200 μg/ml of denatured salmon sperm DNA at 65° C. Filters were hybridized in the same solution with the addition of the PvPR3-5′ insert that was [^{32}P] labeled by the random primer method. Hybridization was carried out at 65° C for 16–24 hr. The filters were then washed at 65° C in 0.1× SSPE, 1% SDS, air-dried, and exposed to X-ray film. Densiometric analysis was carried out using several film exposures in the linear range of film sensitivity using an ISCO gel scanner model 1312 (Isco, Inc., Lincoln, NE).

Genomic DNA from leaves (cultivar Tendergreen) was digested with the indicated restriction enzymes, separated by electrophoresis on 0.8% agarose gels, blotted onto Duralon membranes, and hybridized with the PvPR3-5′ insert according to the manufacturer's instructions. For high stringency hybridization, the blots were washed in 0.1× SSC, 1% SDS at 60° C. For less stringent hybridization, the washes were 0.1×SSC, 0.1% SDS at 42° C.

DNA sequencing. DNA sequences were determined by the dideoxy chain-termination method (Sanger *et al.* 1977) using recombinant M13mp19 phage or pBluescript phagemids as templates. Synthetic oligonucleotides were used as primers to provide the complete sequences of both strands of the DNA. Nucleotide and amino acid sequence alignments and nucleic acid and protein database searches were conducted using IG Suite programs (IntelliGenetics, Inc., Mountain View, CA).

RESULTS

Isolation of PvPR3 cDNA clones. We originally screened a λgtll cDNA library made from bean cell suspension cultures treated with fungal elicitor for 3.5 hr to identify sequences homologous to a rabbit cytochrome P-450 cDNA clone, P-450-4 (Okino *et al.* 1985). A positively hybridizing clone, λ8-2-1, was identified, and the 530-bp insert fragment was subcloned into a plasmid and designated PvPR3-5′. Subsequent sequence analysis revealed that the PvPR3-5′ sequences possessed no significant homology with the P-450-4 coding region. Instead, the cross-hybridization was due to a CT-rich repeat region spanning approximately 60 bp in the PvPR3-5′ clone. This repeat occurs in the 5′ and 3′ untranslated regions of the PvPR3 and P-450-4 transcripts, respectively.

Preliminary characterization indicated that PvPR3-5′ was a partial clone of a 900-bp transcript induced by elicitor treatment of cell cultures. To obtain the complete sequence of the PvPR3 transcript, we used the PCR to amplify the 5′ and 3′ PvPR3 sequences (Fig. 1). We synthesized primers, based on the PvPR3-5′ sequence, which were oriented in both directions. The primers were used with forward and reverse λgtll primers to amplify PvPR3-5′ homologous clones contained within the cDNA library (Friedman *et al.* 1990). We obtained specific 5′ and 3′ products as determined by gel analysis and Southern blotting. Because the 5′ product did not extend beyond the 5′ end of our existing PvPR3-5′ clone, only the 3′ 676-bp product (PvPR3-3′) was subcloned and sequenced. Two independent PvPR3-3′ subclones contained identical sequences and therefore the probability of PCR-generated error in the PvPR3-3′ sequence is exceedingly low.

Regulation of PvPR3 mRNA in suspension cultures by fungal elicitor. We examined the level of PvPR3 mRNA in fungal elicitor-treated bean cell suspensions by northern blot analysis (Fig. 2). A single mRNA species of approxi-

mately 900 bp was hybridized by the PvPR3 probe. The PvPR3 transcript was present within uninduced cells, and the level subsequently increased slightly within 1 hr after elicitor treatment. The mRNA continued to accumulate gradually over a period of 24 hr. After 24 hr, the mRNA was present at 3.5 times the level in unelicited cell cultures as measured by densitometric analysis of autoradiograms. Other experiments have shown that the mRNA accumulates up to 50 hr after elicitor addition (data not shown). To verify that equal amounts of RNA were loaded in each lane, the PvPR3 probe was removed, and the blot was rehybridized with the H1 cDNA clone, which is complementary to a constitutive, abundant RNA (Fig. 2; Lawton and Lamb 1987). The H1 hybridization demonstrates equivalent loading with the exception of the 2-hr lane. This lane contained a slightly higher amount of RNA, which accounts for the slightly stronger PvPR3 signal in this lane.

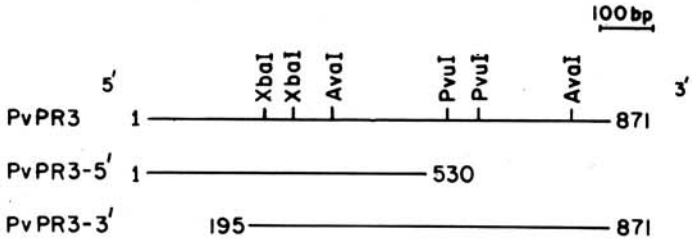

Fig. 1. Restriction map of PvPR3 full-length cDNA based on the sequence of two overlapping cDNA clones shown below, PvPR-5′ and PvPR-3′. The open reading frame occurs between 116 and 526 bp.

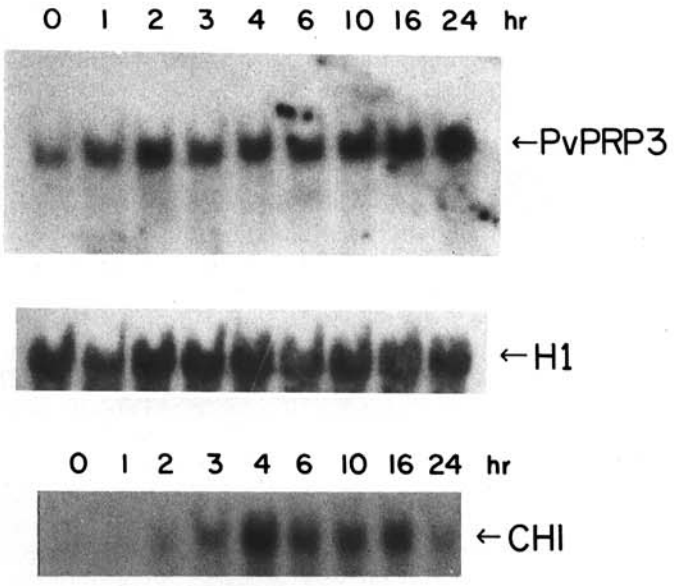

Fig. 2. Time course of PvPR3 mRNA accumulation in suspension cultured cells in response to elicitor. Total RNA was isolated at various times (hr) and analyzed by northern blotting (20 μg per lane). The blot was hybridized with labeled PvPR3-5′ insert. The same blot was then washed and rehybridized with the labeled constitutive clone, H1, to show the relative amount of RNA in each lane. In addition, an identically prepared blot was hybridized with the labeled chalcone isomerase cDNA clone for comparison.

The expression of the PvPR3 mRNA was compared with the expression of chalcone isomerase (CHI) mRNA in the same time course (Fig. 2). CHI catalyzes a step in the biosynthesis of isoflavonoid phytoalexins. The CHI mRNA is undetectable in unelicited cells, then accumulates dramatically to a peak at 4 hr followed by a decline to lower levels. These kinetics are in close agreement with previously published results (Mehdy and Lamb 1987). Thus, the expression of the PvPR3 transcript in elicitor-treated cells is markedly different from the expression of CHI mRNA.

The PvPR3 mRNA induction kinetics are also distinct from induction kinetics shown by transcripts of two members of the hydroxyproline-rich glycoprotein (HRGP) family in bean (Showalter *et al.* 1985; Corbin *et al.* 1987). Using the HRGP cDNA clone Hyp3.6 as a probe, we observed basal levels of the 4.4 and 2.5 kb HRGP mRNAs until 25 hr, followed by a substantial accumulation to stable levels between 30 and 50 hr after elicitor addition (data not shown). Our data showing a delayed onset of HRGP mRNA accumulation are in general agreement with previous studies of elicitor regulation of these HRGP transcripts (Showalter *et al.* 1985; Corbin *et al.* 1987).

Wounding induces PvPR3 mRNA accumulation in etiolated hypocotyls. Because numerous genes induced by fungal elicitor are also wound-inducible, we examined the expression of PvPR3 in mechanically wounded bean hypocotyls (Fig. 3). The PvPR3 transcript was present at relatively low abundance in unwounded tissue and substantially accumulated in wounded tissue. The mRNA accumulated within 2.5 hr of wounding, reached a maximum after 10 hr, then remained at high levels up to 25 hr. Densitometric analysis of the autoradiograms revealed that the

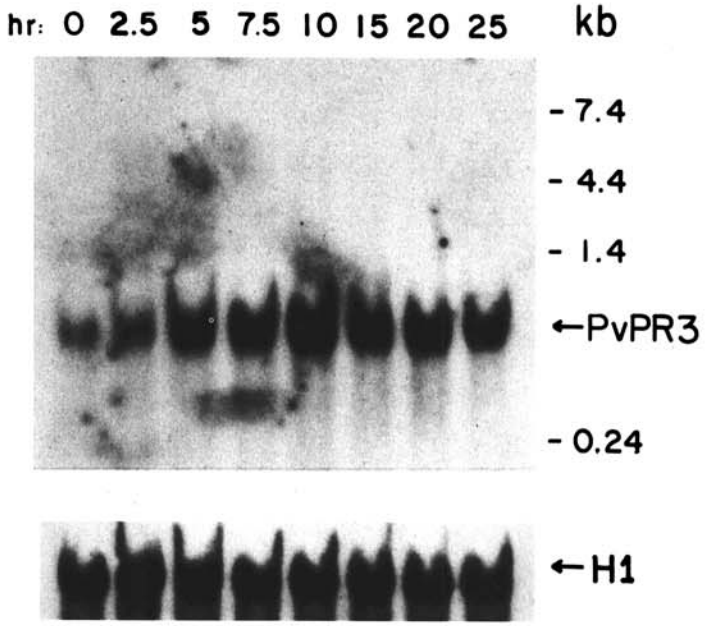

Fig. 3. Time course of PvPR3 mRNA accumulation in wounded segments of bean hypocotyls. RNA (10 μg per lane) was analyzed as described in the legend to Figure 2. The same blot was then washed and rehybridized with the constitutive H1 probe. The migration of RNA molecular weight standards is indicated.

maximal mRNA level (t = 10 hr) is 6.6-fold higher than the level in unwounded tissue (t = 0). Induction of the PvPR3 mRNA has been observed as early as 1 hr after wounding (data not shown). The H1 cDNA probe was again used to demonstrate equivalent loading of RNA in each lane (Fig. 3).

Organization of PvPR3 genes within the bean genome. We analyzed the organization of the PvPR3 sequences in the bean genome by genomic blotting experiments. Genomic DNA was cut with *Eco*RI, *Hin*dIII, *Bam*HI, and *Bgl*II restriction enzymes that do not cut within the PvPR3-5′ cDNA insert used as probe. Figure 4A and B shows that the probe hybridized to single bands in all four digests under high stringency conditions (approximately 5% mismatch permitted). The minor intensity, lower molecular weight hybridizing fragments in the *Hin*dIII digest are not reproducibly observed and were probably due to a trace level of nuclease in the digest. In addition, an *Xba*I digest (Fig. 4B) contained two hybridizing bands as expected, because there are two closely spaced *Xba*I sites in the PvPR3 cDNA clone (Fig. 1). Furthermore, *Kpn*I and *Xho*I digests also contained single hybridizing fragments (data not shown). However, under less stringent conditions (approximately 15–20% mismatch permitted; Bonner *et al.* 1973), the PvPR3 probe hybridized 13–15 bands in *Eco*RI and *Hin*dIII digests (Fig. 4C). This data indicates that PvPR3 exists as a single copy gene within a divergent multigene family consisting of approximately 15 members. Because all of the northern blots were washed at the same high stringency as the genomic blots that showed hybridization to only the PvPR3 gene, it is very likely that the observed patterns of expression reflect only the PvPR3 transcript levels.

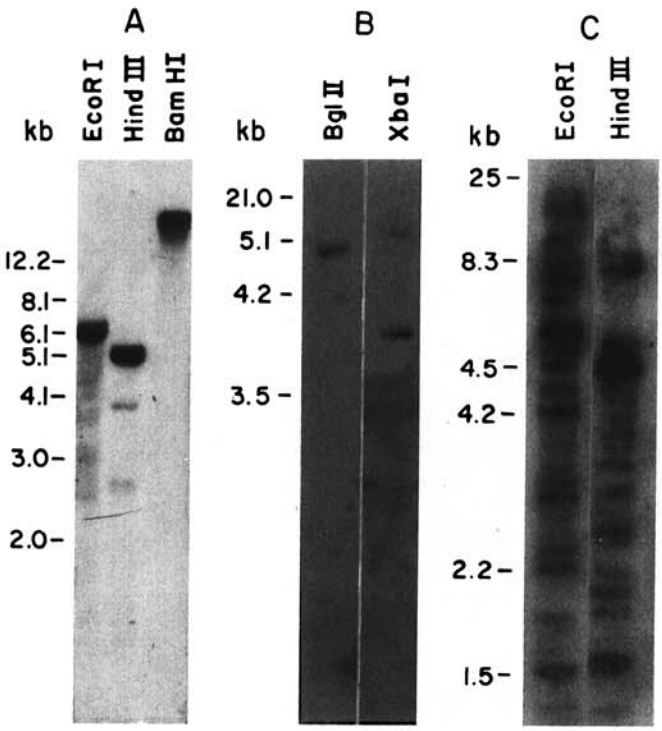

Fig. 4. Genomic blot analysis. Genomic DNA (5 μg per lane) was digested with the indicated restriction enzymes, and the products were size-fractionated on agarose gels. The gels were blotted onto membranes and hybridized with labeled PvPR3-5′ insert. **A** and **B**, the blots were washed at high stringency. **C**, the blot was washed at low stringency. The migration of molecular weight standards is indicated.

Nucleotide sequence of PvPR3 and deduced amino acid sequence. The combined sequence of PvPR3-5′ and PvPR3-3′ is shown in Figure 5. The cloned sequence length (871 bp) represents the full length of the approximately 900-bp transcript. A single open reading frame starts with nucleotide 116 with an ATG start codon and ends at nucleotide 526 before a TGA stop codon. The 5′ untranslated region contains an approximately 60-bp CT-rich repeat region. The long 3′ untranslated region (345 bp) contains a probable polyadenylation signal TATAAA, which is located 28 bp upstream of the 29-base poly(A) tail (Joshi 1987).

The open reading frame encodes a polypeptide of 137 amino acid residues with a predicted molecular weight of 14,950 Da. The putative initiator ATG codon is surrounded by several bases consistent with the plant initiator codon consensus sequence and is the only methionine codon in the open reading frame (Heidecker and Messing 1986). A potential glycosylation site exists at the sequence Asn-Glu-Thr (residues 8–10), which is homologous to the Asn-X-Ser/Thr consensus sequence (Bause 1983).

The hydropathy profile of PvPR3 was analyzed according to the algorithm of Kyte and Doolittle (1982). The protein is largely hydrophilic, including the N-terminal region. There is no evidence of a signal peptide for membrane insertion (Von Heijne 1988). Therefore, PvPR3 is likely to be a soluble, intracellular protein. Of 137 amino acids in the PvPR3 protein, there are 16 acidic (9 Asp, 7 Glu) and 26 basic (15 Arg, 9 Lys, 2 His) amino acids resulting in a strongly basic protein that has an estimated pI of 10.0. The protein is especially rich in alanine (15%) and arginine (11%).

Comparison of PvPR3 with published sequences. The amino acid sequence of the PvPR3-encoded protein was compared with all sequences contained in the Protein Information Resource version 26 and Swissprot 15 protein sequence databases. In addition, the PvPR3 sequence was compared with recently cloned bean PR proteins PvPR1 and PvPR2 (Walter *et al.* 1990) and the potato PR protein *prp1* (Taylor *et al.* 1990). Because bean PvPR1 and PvPR2 were shown to be homologous to a birch pollen allergen (Walter *et al.* 1990), we also searched for homology with a recently published basic rye-grass pollen allergenic protein (Singh *et al.* 1991). Furthermore, the nucleotide sequence of the PvPR3 cDNA clone was compared with sequences in Genbank version 66 and EMBL (March 1991) nucleic acid databases. These analyses revealed no significant homology between PvPR3 and all previously reported sequences.

DISCUSSION

We have identified a gene in *P. vulgaris*, PvPR3, whose corresponding mRNA is regulated by fungal elicitor treatment and wounding. We have operationally defined the

encoded protein as a pathogenesis-related (PR) protein because it possesses a low molecular weight (14,950 Da), is very basic (pI = 10.0), and the transcript is induced during the plant defense response. The structure and regulation of PvPR3 are very distinct from all previously described PR proteins, including two PR proteins recently characterized in bean (Walter *et al.* 1990).

In this paper, we have shown that the PvPR3 transcript gradually accumulates in elicitor-treated suspension cells, with no apparent lag, over a period of 24 hr. This pattern is distinct from the patterns of mRNA accumulation of previously described transcripts that are elicitor-induced. An increased level of CHI mRNA also occurs within 1 hr of elicitor treatment but the mRNA level peaks at 4 hr and subsequently declines somewhat (Mehdy and Lamb 1987; this paper). Transcripts of two families of PR proteins in bean (PvPR1 and PvPR2) accumulate rapidly with maximal levels occurring between 4 and 8 hr (Walter *et al.* 1990). The PvPR3 mRNA induction kinetics also differ from the delayed onset of accumulation of HRGP mRNAs (Showalter *et al.* 1985; Corbin *et al.* 1987). The differential accumulation of the PvPR3 mRNA compared to these other defense gene transcripts suggests that its mechanism of regulation by elicitor treatment is somewhat different. The extended duration of accumulation of its mRNA in cells treated with fungal elicitor suggests that the PvPR3 protein may function at later times during the defense response to pathogens that may complement the early, transient defense responses.

In addition to induction by elicitor, the PvPR3 mRNA rapidly accumulated in wounded hypocotyls of etiolated seedlings. Wounding also results in the induction of the PR protein transcript, pSTH-2 (Matton and Brisson 1989), and the PR-related protein, *wun1* (Logemann and Schell

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5'    1 CAATCCTCTCTCTCTCATTTCCAATCTCAATAATCTCTCTCTCTCTCTCTCTC

 56 TTTCTCTTTCTCTTTCTTCTTCTTCTTCTTCTTTTCTCCTTCCTCACCAAAACGCACTAT

116 ATGGCTCAGAAGGCGCAGAAAAACGACACCGACTTCAAGGTCCCGGAGCCAATCACCCCC

    M  A  Q  K  A  Q  K  N  D  T  D  F  K  V  P  E  P  I  T  P
                          *  *  *
176 TGCGCCACAGCCACCGCCGCCGCAGCAACTTCCATTTCCGAACCATCTAGATTCTTTGAC

    C  A  T  A  T  A  A  A  A  T  S  I  S  E  P  S  R  F  F  D

236 GCTGCAACTCCCGCCACGTCGTCGCGTTCGCCCAAGCGCTCGCTTCCTCTAGAGGACGCT

    A  A  T  P  A  T  S  S  R  S  P  K  R  S  L  P  L  E  D  A

296 GCGAACGCCGACCGGACGGTGGCATCGGAGCCGAAGCGCGCGGTGAACCGGTGCTCGGGA

    A  N  A  D  R  T  V  A  S  E  P  K  R  A  V  N  R  C  S  G

356 TGCCGGCGGCGCGTCGGGCTGACCGGATTCCGCTGTCGGTGCGGTGACCTTTTCTGCGCT

    C  R  R  R  V  G  L  T  G  F  R  C  R  C  G  D  L  F  C  A

416 GAGCACCGGTACACGGACCGCCACGACTGCTCCTACGACTACAAGACCGTCGGGAGAGAG

    E  H  R  Y  T  D  R  H  D  C  S  Y  D  Y  K  T  V  G  R  E

476 GCCATCGCGAGGGAGAATCCCGTCGTGAAGGCCGCGAAGATCGTGAAGGTCTGAAAACCC

    A  I  A  R  E  N  P  V  V  K  A  A  K  I  V  K  V  End

536 CTAAATCGGAGAAGGAGAAAAAACACTTCGATCGATCGCTGAGACGAAACGACGCGAAAC

596 GAAGGCGTTTCGACGCGGTTGGAGATTTAACAGTCTCACGATCGCAGTTAGGTGGTTAAT

656 TTAATTACTCGCAGCGCGTCTCATCTCGGGCGCGTGGGTGGGATCTGGGAAAGTTGTAAC

716 CTGAACGTGAAATTCTCGGTGATCCTTTTTTTTATTTTTTTATGTATAATTTCTTAATTT

776 TTATGTGCTTTTCTTTTTTTTTAATCTCGAGTTGGGATTTATAAATTATTGTGATTATTA

836 TATGATTAAAAAAAAAAAAAAAAAAAAAAAAAAAA                        3'
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Fig. 5. Nucleotide sequence and deduced amino acid sequence of PvPR3. The sequences of two overlapping clones, PvPR3-5′ and PvPR3-3′ were combined to produce the complete sequence. The amino acid sequence is deduced from the single long open reading frame. The sequences marked by asterisks (*) denote a potential N-glycosylation site. A potential polyadenlyation signal is underlined.

1989) in potato tubers. The pattern of changes in the steady-state PvPR3 mRNA level is very similar to the changes observed for several other defense gene transcripts that include phenylpropanoid pathway mRNAs (phenylalanine ammonia-lyase, chalcone synthase, and CHI) and a PR protein, chitinase (Bell *et al.* 1986; Mehdy and Lamb 1987; Hedrick *et al.* 1988).

The increased levels of defense gene transcripts in response to elicitor or wounding have been shown to involve transcriptional activation in nearly all genes analyzed at this level (Dixon and Lamb 1990). Based on this precedent, it is probable that the expression of the PvPR3 gene is also regulated at this level. It will be necessary to perform nuclear runoff experiments to determine the contributions of transcriptional activation and/or regulation of mRNA stability in modulating PvPR3 mRNA levels.

On the basis of our genomic blotting experiments, it appears that PvPR3 exists in the bean genome as a single copy but is a representative of a family consisting of approximately 15 genes. This occurrence of multiple PvPR3-related genes is similar to the multi-gene family organization of most PR protein encoding genes. Genomic blotting experiments showed that the PvPR1 and PvPR2 families together consist of a minimum of 12 genes within the bean genome (Walter *et al.* 1990). In addition, the PR1 gene family in parsley consists of approximately 3–6 genes (Somssich *et al.* 1988), and the tobacco PR1 basic and acidic proteins are encoded by about 16 genes including some inactive pseudogenes (Cornelissen *et al.* 1987).

Comparison of PvPR3 sequences with protein and nucleic acid sequence databases revealed no significant homology with any known sequences. In addition, comparisons with several, recently cloned PR proteins from bean and potato showed no significant homology (Walter *et al.* 1990; Taylor *et al.* 1990). Thus, the PvPR3 product represents a novel PR protein. Although PvPR3 shows no sequence homology with sequences in the databases, the protein has some features in common with two other PR proteins. The protein is rich in alanine (15%) and arginine (11%) and has an isoelectric point of 10.0. The tobacco cluster G PR protein is also basic (pI = 10.4) and contains 16% alanine and 12% arginine (Cornelissen *et al.* 1987). Similarly, the tomato PR protein p14 contains 14% alanine and 10% arginine and has an isoelectric point of 10.7 (Lucas *et al.* 1985).

The properties of the PvPR3 protein are different from previously described bean PR proteins. Bean leaves infected with tobacco necrosis virus were shown to contain acidic proteins of about 16,000 Da (Redolfi *et al.* 1989). In addition, acid-soluble proteins from mercuric chloride-treated bean leaves were analyzed and small (17,000 Da), acidic proteins named PR1 and PR2 were identified (De Tapia *et al.* 1986). PR1 was localized primarily in the extracellular space, whereas PR2 was localized to the cytoplasm. The deduced amino acid sequences of bean PvPR1 and PvPR2 cDNA clones show that these highly related proteins (89% identical) are about 16,000 Da and acidic (Walter *et al.* 1990). Recently, the complete amino acid sequence of the PR2 protein was determined and found to be identical to deduced amino acid sequence of PvPR1 (Awade *et al.* 1991).

Unlike many PR proteins that accumulate extracellularly or within vacuoles (see Bol 1990 for review), the PvPR3 protein is likely to accumulate in the cytoplasm, because a hydrophobic signal peptide cannot be distinguished. PR proteins of unknown functions studied in parsley (Somssich *et al.* 1988), potato (Matton and Brisson 1989), and bean (Walter *et al.* 1990) have also been proposed to accumulate intracellularly. The cellular location of the tobacco cluster G protein is unknown, whereas the tomato p14 protein is postulated to interact with or span membranes (Cornelissen *et al.* 1987; Lucas *et al.* 1985). Purification of the PvPR3 protein and studies of its properties and cellular localization may clarify its functional role during the defense response in plants.

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