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Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases

(viroid/pathogenesis-related protein/defense)

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ABSTRACT **A 69-kDa proteinase (P69), a member of the pathogenesis-related proteins, is induced and accumulates in tomato (*Lycopersicon esculentum*) plants as a consequence of pathogen attack. We have used the polymerase chain reaction to identify and clone a cDNA from tomato plants that represent the pathogenesis-related P69 proteinase. The nucleotide sequence analysis revealed that P69 is synthesized in a preproenzyme form, a 745-amino acid polypeptide with a 22-amino acid signal peptide, a 92-amino acid propolypeptide, and a 631-amino acid mature polypeptide. Within the mature region the most salient feature was the presence of domains homologous to the subtilisin serine protease family. The amino acid sequences surrounding Asp-146, His-203, and Ser-532 of P69 are closely related to the catalytic sites (catalytic triad) of the subtilisin-like proteases. Northern blot analysis revealed that the 2.4-kb P69 mRNA accumulates abundantly in leaves and stem tissues from viroid-infected plants, whereas the mRNA levels in tissues from healthy plants were undetectable. Our results indicate that P69, a secreted calcium-activated endopeptidase, is a plant pathogenesis-related subtilisin-like proteinase that may collaborate with other defensive proteins in a general mechanism of active defense against attacking pathogens.**

In response to pathogen attack, plants orchestrate a multitude of biochemical responses that serve to antagonize the growth of the challenging pathogen as well as to prepare affected plants to resist subsequent pathogenic insults. The active mechanism of defense, as deduced from the study of different pathosystems, include the stimulation of several phenylpropanoid pathways, modification of cell wall properties, and increase synthesis and accumulation of phytoalexins with antimicrobial activity (1). This general response is also accompanied by the accumulation of a characteristic set of proteins, referred to as pathogenesis-related (PR) proteins (2). Genes encoding PR proteins are evolutionarily conserved in the plant kingdom and are induced by different stress situations provoked by biotic or abiotic agents (2, 3). Many PR proteins display enzymatic activities, such as chitinase and β-1,3-glucanase (2, 4) as well as other antimicrobial activities (5) and are thus considered part of the defense arsenal against invading pathogens.

We have employed the viroid-host plant interaction as a system to identify genomic and biochemical responses that become activated following pathogen perception and to gain insights into the mechanisms involved in establishing plant defensive measures (6). Research on the pathogenic mechanism of response of plants to viroid infection has revealed the induction of a set of PR proteins, among which, one unique protein exerts endoproteolytic activity. This protease was identified in tomato plants and was named PR-P69 (7). P69 activity is activated by Ca^{2+}, the enzyme is monomeric with an estimated molecular weight of 69,000, and the protein accumulates in the intercellular spaces of viroid-infected plants (7–9). P69 is also induced in the plant by more complex type of pathogens such as fungi and nematodes (10), as well as by ethylene and salicylic acid, which are considered signal molecules that mediate defense responses in plants (8, 11).

To gain structural and functional information on P69 as a pathogen-induced proteolytic enzyme of tomato plants, we describe the isolation and characterization of a cDNA clone for the viroid-induced P69 proteinase. We show that P69 is synthesized in a preproenzyme form, discuss the predicted structure of the protein, and analyze the pattern of gene expression in healthy and diseased plants. P69 shows molecular similarities with subtilisin-like proteases from animal and yeast sources, notably in the amino acid sequences of the catalytic domains. The potential roles for the P69 in plant-defense reaction are discussed.

MATERIALS AND METHODS

Plant Material. Conditions for growth of tomato plants (*Lycopersicon esculentum*) and the method for inoculation with citrus exocortis viroid have been described (8). Tissues were harvested 4 weeks after inoculation and stored at −80°C.

RNA and PCR Analysis. Procedures for the isolation of RNA and RNA blot analysis have been described (12). PCR was performed in a Perkin–Elmer/Cetus DNA Cycler. A 24-mer degenerate oligonucleotide primer (oP69), complementary to a putative mRNA sequence encoding the last eight amino acid residues of the N-terminal amino acid sequence found in P69 (Gln-Gln-Asn-Met-Gly-Val-Trp-Lys) (10), was designed with the following sequence: 5′-CAA/GCAA/GAAT/CATGGGIGTITGGAAA/G-3′ (I = inosine). The cDNA template for PCR was synthesized from viroid-infected leaf tissue poly(A) RNA or total RNA by using dT12–18 primer and murine leukemia virus reverse transcriptase (Pharmacia). PCR amplification was programmed for 30 cycles, with each cycle consisting of 94°C for 1 min, 50°C for 2 min, and 70°C for 1.5 min. The amplified DNA fragment was isolated from agarose gel, filled in with T4 polymerase, and cloned in *Eco*RV-digested pBluescript (Stratagene).

cDNA Cloning. A λ-ZAP cDNA library was constructed from poly(A) mRNA isolated from viroid-infected tomato

Abbreviations: PR, pathogenesis related; RT, reverse transcription; GST, glutathione *S*-transferase; SPC, subtilisin-related proprotein convertase(s).

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X95270).

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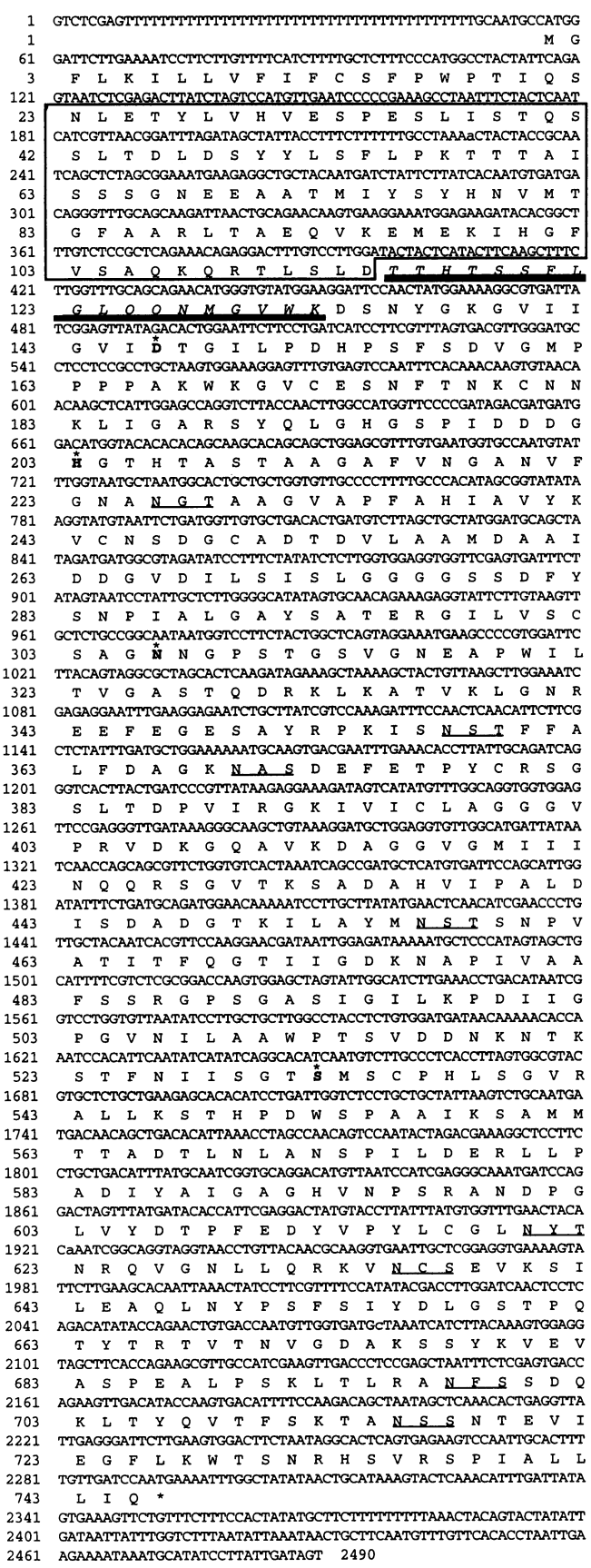

FIG. 1. Nucleotide and deduced amino acid sequences of cDNA encoding tomato P69 proteinase. The catalytically important Asp, His, Asn, and Ser residues are in boldface type and indicated with asterisks. The propeptide domain is boxed. Amino acid residues that are identical to the N-terminal peptide sequence of mature P69 are underlined and shown in italics. Potential consensus sequences for *N*-glycosylation are also underlined. Nucleotide sequence data have been submitted to GenBank, EMBL, and DDBJ data bases as accession number X95270

leaves (12). The library was screened with a radiolabeled DNA fragment obtained by reverse transcription (RT)–PCR as described above, and plaques were isolated by standard techniques as previously described (13). cDNA inserts were excised from phage DNA, cloned into pBluescript, and sequenced by using a T7 polymerase kit (Pharmacia). Nucleotide sequences were analyzed using the University of Wisconsin Genetic Computer Group sequence package (14).

Construction of Fusion Protein, Antibodies Generation and Immunological Studies. A synthetic oligonucleotide (op9) 5′-GTCTAGACACTACTCATACTCC-3′and a T7-specific primer were used as primers to generate a PCR product from the p26 cDNA clone (see *Results*). This PCR product was digested with *Xba*I and *Sal*I and was ligated in the pGEX-KG vector (15). This vector contains the glutathione *S*-transferase (GST), to yield the plasmid pGEX-p26, which was used to produce in *Escherichia coli* a 90-kDa GST–P69 fusion protein (16). The purified GST–P69 fusion protein was injected into rabbits, and the antibodies were obtained (16). Immunoblot analyses using the antibodies generated in this way were performed as described (16). Protein extracts were obtained from leaves in acidic buffer that renders induced PR proteins soluble (8).

RESULTS

Isolation and Characterization of P69 cDNA Clone. The mature P69 isolated from diseased plants contained the 21 N-terminal amino acid sequence (Thr-Thr-Xaa-Thr-Pro/Ser-Ser-Phe-Leu-Gly-Leu-Gln-Gln-Asn-Met-Gly-Val-Trp-Lys) (10). Because the codon usage of tomato nuclear genes is highly biased (17), we took advantage of the most probable codons to design a single oligonucleotide, based on the N-terminal amino acid sequence. The 24-mer oligonucleotide that we designed (5′-CAA/GCAA/GAAT/CATGG-GIGTITGGAAA/G-3′) (I = inosine) was complementary to a putative mRNA sequence encoding the last eight N-terminal amino acid residues of the mature P69. This oligonucleotide, used in combination with oligo(dT) in a PCR on reversed transcribed mRNA (RT–PCR) from viroid-infected tissues, was able to amplify a 2-kb product (data not shown). This PCR-derived DNA fragment was used as a probe to screen a λ-ZAP cDNA library constructed from poly(A) RNA from tomato leaf tissues showing characteristic symptoms of viroid infection. Four independent cDNA clones were obtained after a third round of purification. These clones had 1.3- to 2.4-kb inserts and appeared to have identical nucleotide sequences upon partial nucleotide sequencing. The cDNA clone having the longest insert, designated p26, was used for further analysis.

To test whether the p26 cDNA clone actually encodes the P69 polypeptide, the nucleotide sequence of the 2490-bp insert was determined and confirmed on both strands. The nucleotide and the deduced amino acid sequence are shown in Fig. 1. The cDNA insert of p26 consisted of a 56-bp 5′ untranslated region, a 2235-bp coding region (an open reading frame), a 201-bp 3′ untranslated region, and a 20-bp poly(A) tail. The open reading frame could encode a protein of 745 amino acids. A nucleotide stretch that was complementary to the 24-mer synthetic oligonucleotide probe was localized within the open reading frame at positions 397–453. The deduced amino acid sequence (Fig. 1) derived from the cDNA (positions 115–132) coincides with that of the first 18 amino acids of the mature P69 polypeptide. We assumed that the first ATG in p26 (nucleotides 57–59 in Fig. 1) would be the initiation codon for

translation. This conclusion was based on the following reasons. (*i*) It is the first in-frame ATG from the 5′ end. (*ii*) The length of the p26 insert is in good agreement with the size (2.4 kb) of P69 mRNA (see below). (*iii*) The nucleotide sequences surrounding this first ATG codon matches the consensus sequence conserved for translation initiation in eukaryotes (18).

To verify that the p26 cDNA clone actually encodes the P69 polypeptide we generated an antiserum against the 631-amino acid portion of the mature polypeptide (positions 115–745). This polypeptide was produced in *E. coli* by the expression vector pGEX-KG as an in-frame fusion protein (GST–P69), and upon purification on glutathione-Sepharose was used as immunogen in rabbits. The antiserum generated was immunoreactive toward the induced P69 polypeptide present in acidic crude extracts from viroid-infected tomato plants as well as toward a purified preparation of the viroid-induced P69 proteinase (Fig. 2, lanes 2 and 3). The immunoreactivity was absent in equivalent crude extracts from healthy plants (Fig. 2, lanes 1). Furthermore, the 90-kDa GST–P69 polypeptide produced in *E. coli* was recognized by antibodies previously generated (8) against the P69 proteinase purified from infected plants (data not shown). These results further sustain that the p26 cDNA clone presently described encodes the *bona fide* pathogenesis-related P69 proteinase.

Amino Acid Sequence of P69 Polypeptide. Analysis of the deduced amino acid sequence of P69 using a hydrophobocity algorithm (19) (Fig. 3*A*) showed that after the first methionine (ATG at position 57–59 of the cDNA; Fig. 1) there is an apparent hydrophobic and basic signal peptide sequence of 22 amino as expected for a secreted protein (20) (Fig. 1). Because the N-terminal amino acid sequence in the mature P69 is localized at position 115 in the putative precursor polypeptide (Fig. 1), we assumed that P69 is synthesized in a preproenzyme form with a 22-amino acid signal peptide, a 92-amino acid propolypeptide, and a 631-amino acid mature polypeptide (Fig. 3*B*). The mature P69, after removal of the signal peptide and propeptide, has a predicted molecular weight of 66330 which is roughly coincident with the molecular weight determined biochemically (7). Additionally, the mature polypeptide domain comprised seven potential asparagine-linked glycosylation sites (NXS/T) and a carboxy-terminal region rich in serine and threonine amino acid residues (Fig. 1).

The deduced amino acid sequence of P69 was compared with those of other previously reported proteins in the data bases. This search revealed that different blocks of amino acid sequence in P69 were homologous to those of proteins from the subtilisin serine protease family, like subtilisin BNP′ (21), cucumisin protease from melon (22), or members of the yeast and human proprotein and prohormone subtilisin-like processing proteases [e.g., Kex2 (23), furin (24), PC2/PC3 (25), and PACE (26)]. The amino acid residues (Ser-532, Asp-146, and His-203) could be identified as the residues of the catalytic triad of P69 (Figs. 1 and 3 *B* and *C*) on the basis of sequence similarities. Also, P69 has an Asn residue at a position (Asn-306) that has been found to be highly conserved and catalytically important in the subtilisin family (27, 28). However, and despite the similarities around the amino acids of the active center (Fig. 3*C*), the active Ser-532 residues is displaced when aligned with the rest of the subtilisin members (Fig. 3*B*). There is an insertion of a long sequence (224 amino acid residues) between the stabilizing Asn-306 and the reactive Ser-532 in P69 relative to the other members—e.g., subtilisin BNP′ contains only 65 residues in the corresponding region and Kex2 contains only 80 amino acids. This displacement has also been observed in the cucumisin protease recently cloned from melon fruits (22) and the ag12 protease cloned from nodules of *Alnus glutinosa* (29) (Fig. 3*C*) and thus appears to be the rule for members of the subtilisin-like proteases from plants.

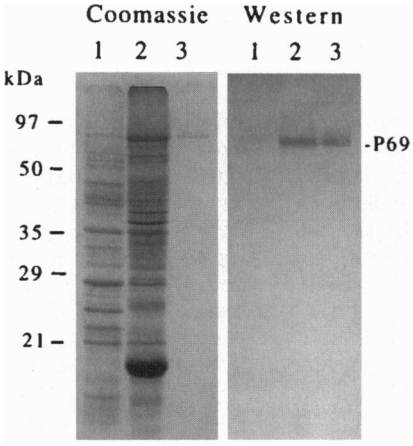

FIG. 2. Specificity of antibodies generated against the bacterial expressed GST–P69 fusion protein toward pathogen-induced P69 proteinase. (*Left*) Comparative SDS/PAGE and Coomassie blue staining of crude acid-soluble proteins recovered from healthy (lane 1) or viroid-infected (lane 2) tomato plants, and P69 proteinase purified from viroid-infected tomato plants (lane 3). (*Right*) Immunoblot of a gel similar to that shown in *Left* using anti-GST–P69 serum.

Northern Blot Analysis of *P69* mRNA Expression in Viroid-Infected Tomato Plants. The differential expression pattern of *P69* was determined in different tissues from healthy and viroid-infected plants by Northern blot hybridization. The filters were hybridized with the radiolabeled RT–PCR-derived DNA product encoding the mature form of P69. The results (Fig. 4) revealed that the level of *P69* mRNA, which has a size of 2.4 kb, is increased markedly in leaf and stem tissue from infected plants, while the corresponding tissues from healthy (mock-inoculated plants) do not show cross-hybridization with the radiolabeled probe. The observed increase in *P69* mRNA level correlated with an extensive increase in protein content and enzymatic activity reported previously in similar tissues from infected plants (8).

DISCUSSION

The data presented here provide structural and functional information on P69 as a pathogen-induced proteolytic enzyme of tomato plants.

The most salient feature of the predicted amino acid sequence of P69 was the presence of domains homologous to the subtilisin serine protease family. The amino acid sequences surrounding Asp-146, His-203, and Ser-532 of P69 are closely related to the catalytic sites (catalytic triad) of the subtilisin-like proteases. In eukaryotes, this class of serine proteases is also refereed to as subtilisin-related proprotein convertases (SPC) of which the yeast *Kex2* (23) is considered the prototype member of this family of proteases. Furthermore, analysis of the different domains in the protein reveal that P69 shares another important feature with the SPC members: the synthesis of P69 polypeptide as a preproenzyme along with the conserved amino acid sequences surrounding the catalytic site. The predicted primary structure of P69 is composed of three domains: a 22-amino acid putative signal peptide that presumably targets the premature enzyme to the outside of the cell as its final destination (via the endoplasmic reticulum) (9), a 92-amino acid propolypeptide that may be responsible for repressing the activity of P69, as stated for the other subtilisin-like proteases (28), and a 631-amino acid mature polypeptide that is the active form of P69 that accumulates *in vivo*. Likewise, the activity of P69 proteinase (7) is similar to that of other subtilisin-like proteinase in its calcium dependence, optimum pH and inhibitor spectrum (23). In plants, two other SPC-like members have recently been cloned and added to this family. One of them, termed cucumisin, is expressed in melon

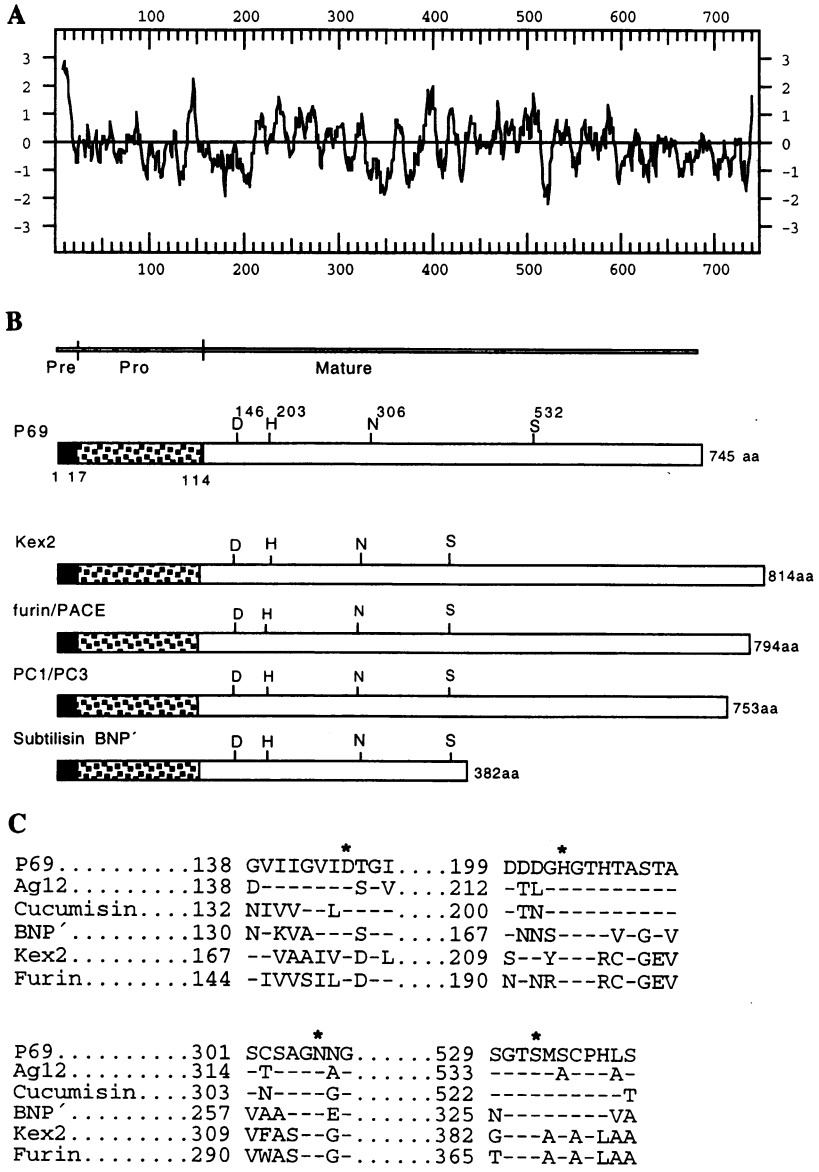

FIG. 3. Structural features and sequence alignment analyses of the P69 polypeptide with subtilisin-like homologs. (*A*) Kyte–Doolittle hydropathy plot. (*B*) Schematic diagram showing structural features and relative lengths of P69, human furin/PACE, yeast Kex2p, human PC1/PC3, and bacterial subtilisin BNP′. Signal sequences are marked with black boxes, prosequences are shown by the shaded boxes, and mature proteins are shown in white. The amino acids forming the catalytic triad in the active site (D, aspartate; H, histidine; S, serine) and the conserved N (asparagine) residue are shown. The distances in the drawings are only approximate. (*C*) Comparison of selected regions of P69 to the amino acid sequence surrounding the active site catalytic residues of ag12, cucumisin, Kex2, subtilisin BNP′, and furin. The relative position of the amino acid segment within each of the proteins is indicated by the numbers to the left of each column. Residues identical to those of P69 are indicated by gaps. Catalytic residues are indicated by asterisks.

fruits and its function remains unknown (22). The other one, termed ag12, appears to be expressed in early stages of actinorhizal nodule formation in *Alnus glutinosa* indicating that the enzyme may be involved in symbiotic processes during plant development (29).

The isolation of the SPC-like *P69* gene from tomato plants provides further evidence that the SPC gene family has been highly conserved throughout evolution of multicellular organisms. Mammalian members of this family of proteases include PC1/PC3, PC2, PC4, PC5/PC6, PACE, and furin (for a review, see ref. 28), whereas the yeast *Saccharomyces cerevisiae* contains a single proprotein convertase gene, *kex2* (23). SPC members have also been describe recently in *Drosophila melanogaster* (30) and *Caenorhabditis elegans* (31). Their role appears to be related with the endoproteolysis of precursor proteins which is required for the synthesis of biological active proteins and peptides in eukaryotes (27). However, definition of the biological significance of individual SPC members has been stymied by the lack of evidence demonstrating substrate specificity. In fact, when the different SPC members are removed from their biological context, many of these endoproteases are able to process the same substrates, raising the question of whether such a functional redundancy exists among the family members *in vivo*. In this regard, restricted expression to particular tissues and compartmentation of individual enzymes to specific cellular locations, apparently influence substrate specificity and activity (31, 32). The different localization of individual SPC enzymes appears to be a function of the structural differences between each family member (31).

The P69 protease described in this paper appears to be the first plant SPC-like protease shown to be induced during pathogenesis. P69 is secreted and accumulates abundantly in the extracellular milieu of leaves and stems cells of infected

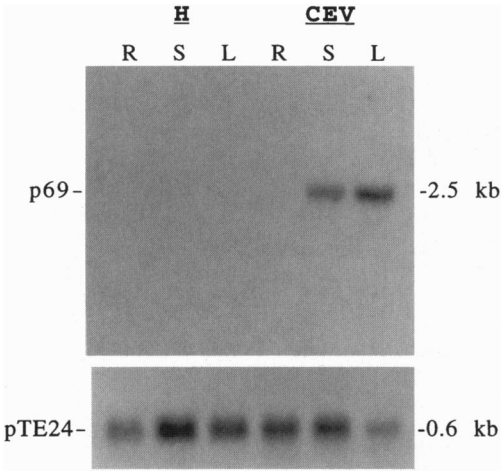

FIG. 4. Expression of P69 mRNA in various tissues from healthy (H) and citrus exocortis viroid (CEV)-infected tomato plants. Ten micrograms of total RNA from each tissue was electrophoresed in duplicate on 1% agarose/formaldehyde gels and probed for *P69* (*Upper*) or the constitutively expressed *pTE24* gene from tomato (*Lower*). R, root tissue; S, stem tissue; L, leaf tissue.

tomato plants (data not shown). Thus, some insight into defining functionality for P69 can be obtained from its cellular localization. Being extracellular, P69 could modulate the interaction of the cell surface with extracellular environment either directly, for example, by localized and directional degradation of the extracellular matrix, or indirectly by activating signal transduction pathways. There is overwhelming evidence that the modification of protein component of the extracellular matrix by proteases is of critical importance for the normal functioning of animal cells (33). Furthermore, increasing evidence demonstrates that cell surface localization of proteinases and proteolytic degradation of the extracellular matrix is a common cellular strategy by which cells initiate phenotypic changes during different developmental processes and in pathological conditions (34). During these cellular processes, an extensive remodeling of the extracellular matrix is brought about by the concerted activation of genes encoding distinct proteolytic enzymes that recognize and degrade pericellular substrates and in turn activate or influence a number of different cellular processes which may form the underlaying basis of certain forms of disease (34, 35). From a functional standpoint, all the evidence suggests that finely tuned proteolytic balance is critical for the normal cell functioning, and that alteration of this balance results in the disruption of the normal processes and aberrant cell behavior. In plants, information regarding remodeling of the extracellular matrix and characterization of proteases that participate in such processes, either under normal or pathological situations, is scant. The availability of the *P69* gene presently described offers unique opportunities to test whether or not this protease participates in any of the above mentioned cellular processes. In this regard, a protein of the plant extracellular matrix, termed LRP, which potentially could mediate signaling processes in tomato plants, is proteolytically processed in viroid-infected tomato plants by the P69 proteinase (unpublished data). This result emphasizes the potential role of P69 in signal transduction by recognizing, processing, and activation of signaling precursor molecules that could eventually modulate the plant defense response. Likewise, an antiserum against a Kex2-like protease from *Drosophila* immunodecorates a protein band of about 60 kDa in Western blots from tomato plasma membrane proteins (36). This ≈60-kDa protein has been proposed to act in the recognition and processing of systemin, the plant translocated peptide hormone mediating signaling processes during wound response in plants. The *P69* gene presently described is not induced by wounding (data not shown), and we do not know yet if P69 corresponds biochemically to the recently identified prosystemin processing enzyme. This possibility broadens the perspective of P69 or other SPC-like proteases as a pathogen-induced protease that could eventually participate in different signaling processes.

At present, very little is known about the expression of plant protease genes and their role in response to pathogen attack. Recently, a gene encoding a leucine aminopeptidase that is activated during the defense response of tomato plants to *Pseudomonas syringae* has been described (37). Thus it is possible that coordinate expression of genes encoding different proteases, like leucine aminopeptidase or the presently described P69, are induced during pathogen attack and may reflect common strategies evolved by plants to defend against pathogens, either acting as a first defense barrier with capability to degrade proteins of invading pathogens or acting in the posttranslational modification of proteins that participate in the defense response. Whatever the function of P69 is, its identification as a conserved plant subtilisin-like protease open new avenues for the better understanding on how plants respond to pathogenic insults. The availability of the P69 cDNA clone will allow us to search for additional members of subtilisin-like proteases involved in pathogenesis and also express P69 in transgenic plants and test its biological role during the defense response.

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