Research Note

Cloning and Expression Analysis of a Viroid-Induced Peroxidase from Tomato Plants

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Differential hybridization was used to detect transcripts induced both by ethylene and by viroid infection in tomato plants. A cDNA clone encoding a putative peroxidase was isolated and characterized. DNA sequencing revealed high homology with a lignin-peroxidase from tobacco. Northern blot analysis showed specific induction of this peroxidase gene in viroid-infected plants. An increase in the level of mRNA accumulation is obtained by ethylene treatment, reinforcing the idea that ethylene is a mediator in the response of tomato plants to viroid infection.

Additional keywords: CEV, defense, pathogenesis, PR proteins.

Being sessile, plants have evolved a large array of active defense mechanisms, many of which are accompanied by *de novo* transcription of plant defense genes (Dixon and Lamb 1990). Collectively, these induced reactions are thought to contribute to an active defense mechanism of the plant known as "induced resistance."

We are interested in the response of plants to viroid infection and, in particular, the mechanisms and signals that mediate this response (for a review, see Conejero *et al.* 1990). The viroid infection process culminates in systemic spread of the viroid, concomitant with the appearance of a developmental syndrome in the plant. This syndrome is characterized by stunting of the plant (internode shortening), epinasty, and rugosity of the leaves, and abnormal development of roots and vascular tissues (for a review, see Semancik and Conejero 1987). We have shown that the plant hormone ethylene plays a pivotal role in this process and that most of the physiological, biochemical, and developmental alterations induced by viroid infection can be mimicked when this hormone is applied exogenously to the plant (Conejero *et al.* 1990). That ethylene probably mediates pathogenesis has also been demonstrated in many different plant-pathogen interactions (Boller 1991).

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In our search for host cell factors involved in the pathological response of tomato plants to viroid infection, we have isolated several cDNA clones of mRNAs that are altered as the viroid infection progresses and induces the developmental syndrome in the plant.

Here we report on the molecular cloning, sequence analysis, genomic organization, expression, and some functional implications of one of these cDNA clones. This clone represents a gene, designated *cevi-1* (for *c*itrus *e*xocortis *v*iroid *i*nduced), that codes for a lignin-peroxidase (EC 1.11.1.7, donor:hydrogen-peroxidase oxidoreductase).

We have prepared two cDNA libraries using poly $(A)^+$ RNA from tomato leaves treated with ethephon (an ethylene-releasing compound) or from tomato leaves infected with citrus exocortis viroid (CEV) and showing characteristic symptoms of viroid infection. The ethylene-induced library was prepared from leaves treated for 72 hr with 1 mM ethephon (Vera and Conejero 1990). Differential screening, using radiolabeled first-strand cDNA probes prepared from the equivalent poly$(A)^+$ RNA either from healthy leaves, CEV-infected leaves, or leaves treated with ethephon, identified induced cDNAs common to both viroid infection and ethephon. Of 3×10^4 recombinant phages screened from the non-amplified ethephon library, 104 were differentially induced by ethylene. Of these 104 clones, 34 were also shown to be viroid inducible. These 34 independent clones were studied further for sequence similarities by cross-hybridization and nucleotide sequencing. The clone designated pTE35 was chosen for further studies.

Sequence analysis of the pTE35 cDNA clone and database comparisons revealed strong homology with a lignin-peroxidase from tobacco (Lagrimini *et al.* 1987). Sequencing of pTE35 cDNA revealed that it was an incomplete clone, lacking the 5′-end region. We used the cDNA insert of this clone to screen the viroid-specific nonamplified cDNA library. Seventeen related clones were obtained, and the largest, pTE 35-7, was selected for further analysis.

The complete nucleotide sequence of pTE 35-7, designated cevi-1 peroxidase (from citrus exocortis viroid-induced peroxidase), is presented in Figure 1 with its deduced amino acid sequence. The cevi-1 peroxidase protein contains 324 amino acids and has an M_r of 34,635. The cDNA sequence predicts that the protein is synthesized as a preprotein with a hydro-

phobic signal sequence of 22 amino acids (Fig. 1). The signal peptide may direct targeting of the protein to the extracellular space, as described for the lignin-peroxidase from tobacco (Lagrimini *et al.* 1987). The mature protein contains three potential N-glycosylation sites (Asn, X, and Thr/Ser, at residues 13, 128, and 183, respectively). The predicted amino acid sequence gives a pI of 4.35 for the mature protein. The cDNA is 1,196 bp long, containing 15 bp of the 5′-nontranslated leader sequence and 196 bp of 3′-untranslated sequence before the poly(A) tail. This 3′ sequence contains a consensus polyadenylation signal (AATAAA, Fig.1).

The amino acid sequence derived for the cevi-1 peroxidase was compared with the amino acid sequence of the tobacco lignin-peroxidase (Lagrimini *et al.* 1987). The two proteins have 80% identical amino acids. The sequences of the two polypeptides were aligned with gaps to allow the best fit for each protein; their sequences are compared in Figure 2.

To study the expression of the cevi-1 peroxidase gene, RNA was isolated from the apex of tomato plants at different times after inoculation with CEV and subjected to Northern blot analysis to determine the level of accumulation of transcripts for the cevi-1 peroxidase (Fig. 3A). This study revealed that tomato plants induced accumulation of cevi-1 peroxidase mRNA 2 wk after inoculation with the viroid, at which time characteristic symptoms of viroid infection were becoming visible in the apical leaves. Even after severe symptoms had emerged in the infected plant (4 wk after inoculation), the steady-state level of accumulation of transcripts for the cevi-1 peroxidase remained constant. RNA samples from mock-inoculated healthy plants of the same age did not show accumulation of the transcript (data not shown). This pattern of expression of cevi-1 peroxidase mRNA in viroid-infected plants was verified several times with plants grown at different times of the year. In every case, a period of 2 wk from inoculation with viroid was necessary for full expression. This timing of expression coincided with that observed for another viroid-inducible gene from tomato encoding a PR-1 protein (Tornero *et al.*, in press). Furthermore, analysis of different plant tissues from healthy or viroid-infected plants revealed that cevi-1 peroxidase mRNA is

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1     GGCACGAGATCAATAATGTCTTTTTTGAGATTTATTTTTCCACTTTTCTTCTTGATTTCA   60
                M  S  F  L  R  F  I  F  P  L  F  F  L  I  S
61    ATTTTCGTAGCATCGAATGCTCAATTAAGTGCAACATTTTACGCATCTACTTGCCCTAAT  120
      I  F  V  A  S  N  A  *Q  L  S  A  T  F  Y  A  S  T  C  P  N
121   GTTACCGAAATTGTACGTGGTGTCATGCAACAAGCCCAAAGTACCGTAGTTCGTGCTGGT  180
      V  T  E  I  V  R  G  V  M  Q  Q  A  Q  S  T  V  V  R  A  G
181   GCTAAAATTATTCGTCTTCATTTTCACGATTGCTTTGTTAATGGTTGTGATGGATCTCTT  240
      A  K  I  I  R  L  H  F  H  D  C  F  V  N  G  C  D  G  S  L
241   TTGCTAGATAATGCAGCTGGGATTGAAAGTGAAAAAGATGCAGCTTCAAATGTTGGTGCT  300
      L  L  D  N  A  A  G  I  E  S  E  K  D  A  A  S  N  V  G  A
301   GGAGGATTTGATATTGTGGATGATATTAAAACTGCGTTGGAAAACGTGTGTCCTGGCGTT  360
      G  G  F  D  I  V  D  D  I  K  T  A  L  E  N  V  C  P  G  V
361   GTTTCTTGTGCTGATATTTTAGCTCTTGCATCTGAAATTGGAGTTGCCTTGGTTGGAGGT  420
      V  S  C  A  D  I  L  A  L  A  S  E  I  G  V  A  L  V  G  G
421   CCAACATGGCAAGTTCTTCTAGGGAGAAGAGATAGCTTAACAGCAAATAGAAGTGGAGTT  480
      P  T  W  Q  V  L  L  G  R  R  D  S  L  T  A  N  R  S  G  V
481   GATAGTGATATCCCAACTCCATTTGAAAGCCTTGATGTTATGAGACCACAATTCACCAAC  540
      D  S  D  I  P  T  P  F  E  S  L  D  V  M  R  P  Q  F  T  N
541   AAGGGAATGGATATAACTGATCTCGTTGCTCTATCAGGTGCACATACATTTGGAAGGGCA  600
      K  G  M  D  I  T  D  L  V  A  L  S  G  A  H  T  F  G  R  A
601   AGATGTGGCACATTTCAACAAAGACTTTTCAATTTTAGTGGAAGTGGTAGCCCTGATCCA  660
      R  C  G  T  F  Q  Q  R  L  F  N  F  S  G  S  G  S  P  D  P
661   ACAATAAACTCAACTTATTTGCCAACACTACAAGCAACTTGTCCACAAGGTGGAAACAAT  720
      T  I  N  S  T  Y  L  P  T  L  Q  A  T  C  P  Q  G  G  N  N
721   GGGAATACTTTTGAAAATCTTGATAAAACAACTCCAGATAATTTTGATAATGACTATTAC  780
      G  N  T  F  E  N  L  D  K  T  T  P  D  N  F  D  N  D  Y  Y
781   ATAAATCTTCAAAATCAAGAAGGTCTACTTCAAACTGATCAAGAATTATTTTCGACATCG  840
      I  N  L  Q  N  Q  E  G  L  L  Q  T  D  Q  E  L  F  S  T  S
841   GGATCCGATACAATCGCGATAGTGAATCGTTACGCGAGTAGTCAGAGCCAATTTTTCGAT  900
      G  S  D  T  I  A  I  V  N  R  Y  A  S  S  Q  S  Q  F  F  D
901   GATTTTGCTAGCTCGATGATTAAATTGGGAAATATCGGTGTGTTAACGGGTACTAATGGA  960
      D  F  A  S  S  M  I  K  L  G  N  I  G  V  L  T  G  T  N  G
961   GAGATTAGGACTGATTGTAAGAGGGTTAATTAGTTGATTAGGAGGGGTTTATAAGTTATA 1020
      E  I  R  T  D  C  K  R  V  N  ●
1021  ATATATGCGTGATAAATTGTGTTTGTGTTTTCGTACGAAAAAGAGAGATATTATAATGTC 1080
1081  TTGATCAAGTGTAATTTTGCTTTTGTTGAAGTGTAATATGTGCTAAAATAGCAATCCTAA 1140
1141  ATAAATAAGAATGATATGCTGAAAAGCAATACCAAGTTTTATCATTTAAAAAAAAA  1196
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Fig. 1. Nucleotide sequence of the tomato cevi-1 peroxidase cDNA clone. The predicted amino acid sequence is given above in single-letter code. The first amino acid of the mature protein is indicated by an asterisk and the TTA stop codon at the end of the deduced protein sequence by a dot. N-glycosylation sites are underlined; the poly (A) signal is double-underlined. DNA sequencing was performed on both strands as described (Hattoni and Sakaki 1987).

expressed most abundantly in leaves from infected plants. Stems from infected plants also showed a moderate level of expression, whereas roots showed only weakly detectable cevi-1 peroxidase mRNA accumulation (Fig. 3B). Hybridization to RNA from equivalent tissues from healthy tomato plants did not reveal any of this transcript (data not shown). Only stems from healthy plants revealed a weak hybridizing band after prolonged exposure (7 days) of the blot (not shown). Stem-specific expression, albeit at low levels, has also been described for the homologous lignin-peroxidase from tobacco in nonstressed plants (Lagrimini *et al.* 1987).

Many of the developmental, physiological, and biochemical alterations produced by viroid infection can be reproduced by exogenous application of ethephon to healthy plants (Conejero *et al.* 1990). To determine the effect of ethylene on expression of cevi-1 peroxidase, leaves from healthy tomato plants were treated continuously with 1 mM ethephon, and RNA was isolated at different times after treatment. After hybridization of Northern blots with a radiolabeled cevi-1 peroxidase probe, increased levels of cevi-1 peroxidase mRNA were detected 6 hr after ethephon treatment (Fig. 3C). The level reached a plateau by 48 hr. The kinetics of induction are similar to that reported for a senescence-related peroxidase gene from cucumber cotyledons after ethephon treatment (Morgens *et al.* 1990).

To determine the complexity of the *cevi-1* peroxidase gene, genomic DNA from tomato leaves was digested to completion with different restriction enzymes and subjected to Southern hybridization analysis (Fig. 4). Hybridization to the genomic DNA was done at high stringency (65° C, as described by Church and Gilbert 1984). Two major hybridization bands were observed, suggesting the existence of at least two copies of the *cevi-1* peroxidase gene in the tomato genome.

Plant peroxidases are involved in a variety of physiological and biochemical phenomena: polymerization of phenolic monomers into lignin and suberin (Grisebach 1981; Cottle and Kolattukudy 1982), wound-healing (Espelie *et al.* 1986), polysaccharide cross-linking (Fry 1986), regulation of active auxin levels (Hinnman and Lang 1965), cross-linking of proline-rich proteins and extensin monomers (Everdeen *et al.* 1988; Bradley *et al.* 1992), cell elongation (Goldberg *et al.* 1986), and pathogen defense (Vance *et al.* 1980; Hammerschmidt *et al.* 1982). In all these physiological processes, the isozyme pattern of peroxidase is often complex and has posed problems in attempts to understand the specific function of these enzymes *in vivo* and their specific role in plant growth and adaptation to the enviroment (Welinder 1992).

In this article, we describe the molecular cloning of a tomato peroxidase (cevi-1 peroxidase) whose transcript level is up-regulated by viroid infection. The deduced amino acid sequence of the cevi-1 peroxidase shows very high homology with the amino acid sequence of a lignin-peroxidase from tobacco (Lagrimini *et al.* 1987), the role of which in lignification is under study using transgenic plants (Lagrimini *et al.* 1990). Sequence comparisons with other plant peroxidases do not reveal homologies greater than 50%. In particular, a weak

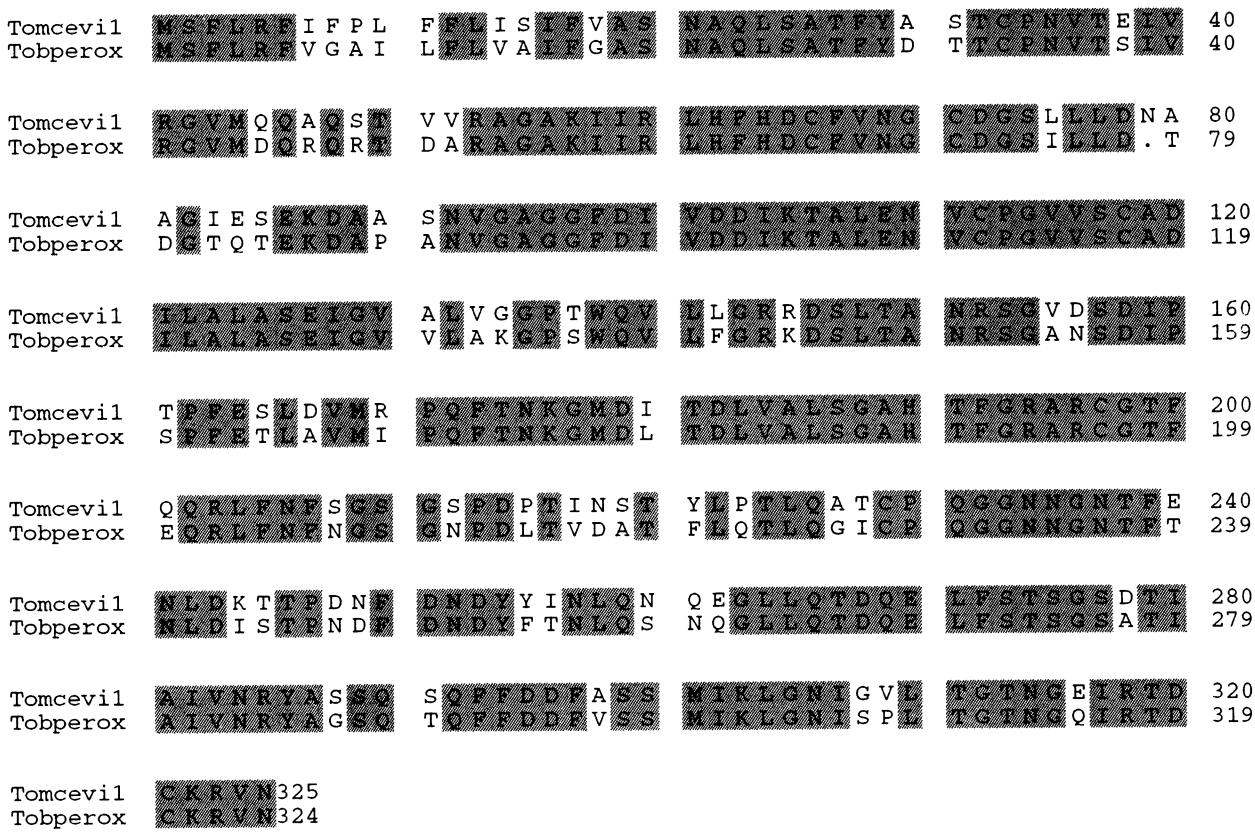

Fig. 2. Comparison of the deduced amino acid sequences of tomato cevi-1 peroxidase and tobacco lignin-peroxidase (Lagrimini *et al.* 1987). The sequences were aligned by BESTFIT. Amino acid positions identical in the two sequences are shaded. A gap is shown by a dot. The arrowhead indicates the signal peptide cleavage site.

homology (35% identity) was found between the cevi-1 peroxidase and the anionic peroxidase from potato (Roberts *et al.* 1988). It is noteworthy that the tomato cevi-1 peroxidase shows only 29% identity with the anionic peroxidases (TAP1 and TAP2) from tomato, whose role in suberization and defense against bacterial infection has been established (Roberts and Kolattukudy 1989; Mohan and Kolattukudy 1990). Many of the cytopathological changes occurring upon viroid infection are related to alterations in cell wall properties (e.g., cell wall aberrations in the form of undulations and irregular thickening, increased cell-cell adhesion, inhibition of cell wall loosening and extensibility, and increased content of hydroxyproline and 5′-linked arabinosyl residues, as markers for hydroxyproline-rich glycoproteins and arabinogalactan proteins, respectively) (Wang *et al.* 1986; Semancik and Conejero 1987). These findings, together with the fact that cell wall components are the targets for the action of secreted peroxidases (Fry 1986), favor the hypothesis that cevi-1 peroxidase is an important cellular factor mediating these viroid-specific cytopathological alterations. We have proposed that loss of the juvenile condition of target cells required for initiating a compatible interaction is the basis for nonspecific resistance to viroid infection (Semancik and Conejero 1987; Conejero *et al.* 1990). The potential function of cevi-1 peroxidase on cell wall hardening suggests that it might be implicated in this type of resistance.

Note also the additional activity of different peroxidases in the oxidative inactivation of the plant hormone indole-3-acetic acid (IAA) (Hinnman and Lang 1965). In fact, preliminary observations indicate that the presence of a viroid enhances the total peroxidase activity in tomato and induces the appearance of an anionic isoenzyme that possesses IAA oxidase activity (Rodriguez 1986). The oxidation of auxin by a viroid-induced peroxidase could explain why viroid-infected plants show delayed and reduced rooting and cell wall extensibility (Semancik and Conejero 1987). These two phenomena are characteristic responses of plant tissues to auxin imbalance. This model gains support if we consider that the presently described peroxidase is induced by ethylene, which by itself is able to block the response of tissue to auxin with a concomitant increase in cell wall hardening (Sadava and Chrispeels 1973), as is true in viroid-infected plants.

Our result with viroid infection and ethylene treatment inducing cevi-1 peroxidase mRNA parallels that for different pathogenesis-related (PR) proteins (Tornero *et al.*, in press). This suggests that cevi-1 peroxidase and PR proteins are regulated in a coordinated fashion as a result of pathogen attack or ethylene treatment. Such synchronized induction may be important considering that peroxidases, and other defense-related proteins, have a role in containing and deterring any additional pathogen challenge (Mohan and Kolattukudy 1990). Thus, it is not unreasonable to consider the inducible cevi-1 peroxidase as a new member of the PR proteins, which are proposed to play a role in the defense response (Bol *et al.* 1990).

The cDNA probes developed in this work will enable us to study the activation of genomic sequences encoding this peroxidase upon viroid infection. Studies of this type with different PR genes are already in progress, and it will be interesting to compare the 5′ regulatory regions of the coinduced peroxidase and PR protein genes.

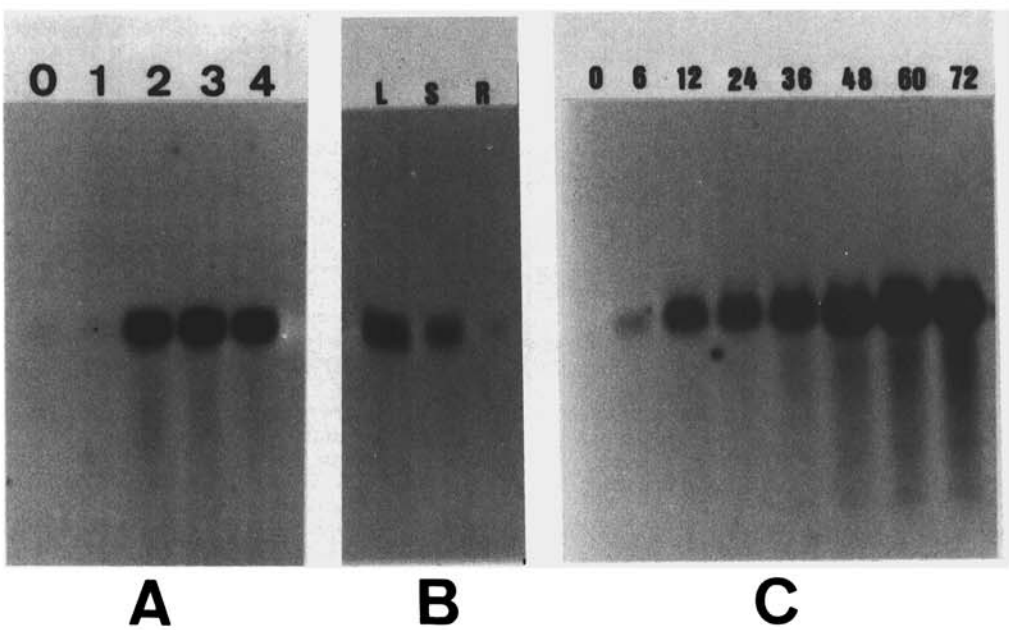

Fig. 3. Expression analysis of cevi-1 peroxidase. **A**, Northern blot analysis of tomato cevi-1 peroxidase transcript at various times postinoculation with citrus exocortis viroid. Total RNA (15 μg per sample) was isolated from apical leaves of tomato plants at different times after inoculation with citrus exocortis viroid. Numbers on top indicate weeks after inoculation. **B**, Accumulation of cevi-1 peroxidase mRNA in different plant tissues from viroid-infected plants. Total RNA was isolated from leaves (L), stems (S), or roots (R) from viroid-infected plants 4 wk postinoculation. RNA (15 μg) from each tissue was electrophoresed and blotted. **C**, Time-course of induction of cevi-1 peroxidase mRNA in tomato leaves treated with ethephon. Each lane contained 15 μg of total RNA extracted from leaves after continuous incubation in 1 mM ethephon. Numbers on top represent time in hours. RNA was purified as described (Logemann *et al.* 1987), fractionated on agarose gels containing formaldehyde, and blotted onto Nytran membranes as described (Maniatis *et al.* 1982). Blots were probed with a ^{32}P-random-primed purified pTE35-7 insert containing the entire cDNA clone. Hybridization and washing of filters were as described (Church and Gilbert 1984).

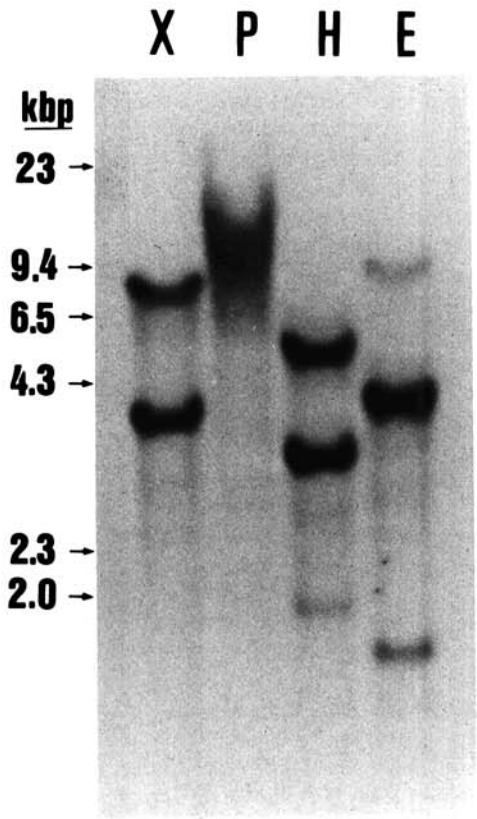

Fig. 4. Southern blot of digested genomic tomato DNA. DNA (10 µg) wa digested with restriction enzymes *Xba*I (X), *Hind*III (H), *Pst*I (P), o *Eco*RI (E); electrophoresed; blotted; and hybridized to a labeled cevi-peroxidase cDNA probe. DNA was isolated from tomato leaves a described (Rogers and Bendich 1988).

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