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Characterization of Defense-Related Genes Ectopically Expressed in Viroid-Infected Tomato Plants

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Differential hybridization was used to isolate genes induced by viroid infection in tomato plants. Four new cDNA clones encoding a peroxidase, a desaturase-like enzyme, a lipoxygenase, and a proteinase inhibitor, were selected and characterized. All of these genes display a characteristic expression pattern, showing constitutive expression in roots of healthy plants and being ectopically activated in aerial tissues upon viroid infection and ethephon treatment. Possible functions for these genes in the viroid-tomato interaction are proposed. The existence of an integrated program that compiles developmental and defense-related responses is also suggested to explain the characteristic expression pattern detected for these genes as well as for other defense-related genes.

Additional keywords: host-pathogen interaction, proteinase inhibitor II.

Plants have developed an array of inducible defense mechanisms thought to help them ward off attacking pathogens. These mechanisms are generally constructed by transcription of a large number of plant defense-related genes. Among them, genes important for cell wall reinforcement, genes coding for key enzymes of the phenylpropanoid pathway and phytoalexin synthesis, or those encoding pathogenesis-related proteins (PR proteins), have been widely characterized in a variety of pathosystems (Dixon and Lamb 1990). Collectively, these induced reactions contribute to an active defense mechanism of the plant known as "induced resistance."

As part of a long-term aim of better understanding the defense reactions against pathogens in tomato, our laboratory has been concentrating on the response of tomato plants to viroid infection, and—in particular—on what kind of mechanisms and signals mediate this response (Conejero et al. 1990). The viroid infection process culminates in systemic spread of the pathogen, accompanied by the induction of a resistant character in affected plants to subsequent pathogenic attacks (Semancik and Conejero 1987). This resistance is presumably the result of the combination of a number of active defense responses that include the transcription of several genes encoding proteins involved in these responses (Conejero et al. 1990; Domingo et al. 1994; Tornero et al. 1994; Vera et al. 1993). Concomitantly, the appearance of a developmental syndrome (e.g., severe stunting of the plant, epinasty and rugosity of the leaves, and abnormal development of roots and vascular tissues) is another characteristic of viroid-infected plants and indicates that the developmental alteration and induction of defense-related genes are coordinately induced and integrated within a complex programmed response.

Evidence has been obtained for the involvement of the hormone ethylene in many of the viroid-induced responses. Endogenous levels of ethylene increase in viroid-infected plants; this is consistent with ethylene's association with the systemic responses elicited in response to viroid infection (Conejero et al. 1990). Moreover, most of the physiological and developmental alterations induced by viroid infection can be mimicked when ethephon (an ethylene-releasing compound) is exogenously applied to the plant (Conejero et al. 1990 and references therein).

In an attempt to define whether the observed correlation between viroid-infection and ethylene activation is specific for certain genes (e.g., *PR* genes) or represents a more general mechanism that the plant uses for activation of numerous genes, we set out to identify additional genes showing altered expression patterns in viroid-infected plants. We report the isolation, by differential screening, of four additional viroid-induced and ethephon-responsive genes and characterize their expression. These new genes appear to be constitutively expressed in certain organs of healthy plants (e.g., roots) and become expressed ectopically in other tissues of the plant upon infection with citrus exocortis viroid (CEV). In the context of viroid-induced defense responses, the mode of expression of these newly isolated genes and the putative role of the encoded gene products indicate that they could be important cellular factors for manifestation of viroid-induced resistance and/or pathological symptoms in the plant. Thus, their characterization provides additional information for dissection of the signal pathways for induction of defense responses in the plant upon viroid infection.

To isolate citrus exocortis viroid-inducible (CEVI) cDNAs clones from tomato that are also ethylene-responsive, we constructed a λ-ZAP-tomato cDNA library, using poly(A) RNA from viroid-infected plants, and screened this library in triplicate with radiolabeled cDNA probes prepared from RNAs from control, viroid-infected, and ethephon-treated tomato leaves (Vera et al. 1993). Several clones were obtained which showed positive hybridization with viroid-infected and

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Nucleotide sequence data have been submitted to GenBank, EMBL, and DDBJ as accession numbers: X94943 for *CEVI16*; X94944 for *CEVI19*; X94945 for *CEVI34* and X94946 for *CEVI57*

ethephon-treated probes, but very low or no hybridization with the control probe prepared from healthy untreated leaves. The cDNA clones CEVI16, CEVI19, CEVI34, and CEVI57 were transfered to pBluescript and characterized.

To define the expression pattern of the different clones during normal plant development, radiolabeled probes corresponding to these genes were hybridized to mRNAs from leaf, stem, and root tissues of healthy plants (4 wk after germination). Northern blot analysis with these probes revealed a pattern of constitutive accumulation of all four mRNA in root tissues (Fig. 1). In addition, *CEVI19* showed high levels of expression in stem tissues. The expression of all four clones was undetectable in leaves. The effect of viroid infection on transcript levels of the four clones was investigated in parallel

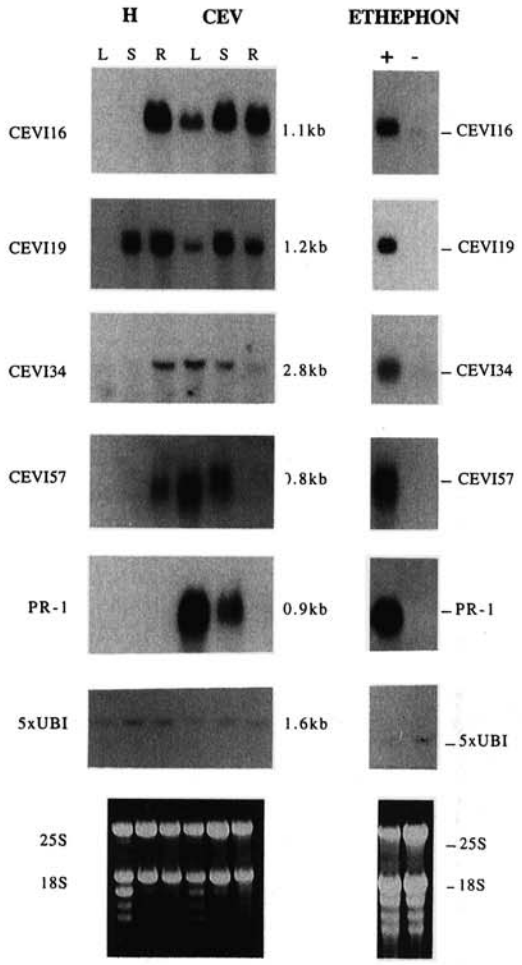

Fig. 1. Northern hybridization analysis showing differential expression of the mRNAs hybridizing to cDNAs CEVI16, CEVI19, CEVI34, and CEVI57. (Left) Hybridization to RNA samples isolated from different tissues (leaf (L), stem (S) and root (R)) of tomato plants 4 weeks after inoculation with citrus exocortis viroid (CEV) or from mock-inoculated healthy control plants of the same age (H). (Right) Hybridization to RNA isolated from leaf tissue at 60 h after treatment with 1 mM ethephon. Northern blots were hybridized with the cDNA inserts described above, with the PR-1b1 cDNA described previously (Tornero et al. 1994), or with a cDNA clone encoding a pentaubiquitin (unpublished results) that is constitutively expressed. Ethidium bromide stained gels is shown to verify equal loading of RNA. Hybridization and washes of filters were done at 70°C with the Church and Gilbert (1984) hybridization solution.

using mRNA extracted from viroid-infected plants of the same age and grown under identical greenhouse conditions. The protocol for inoculation with CEV has been described previously (Vera and Conejero 1989). In contrast to controls, plant leaf and stem tissues showed a large accumulation of the mRNA corresponding to all clones in leaf and stem tissues following viroid infection (Fig. 1). Some marked differences in the general pattern of expression of these genes in viroid-infected tissues were observed. *CEVI34* and *CEVI57* are expressed in aerial parts of the infected plant (leaf and stem tissue) but their normal constitutive expression in roots seems to be repressed. *CEVI16* follows a similar induction in leaves and stems of infected plants but remains expressed in healthy roots. Likewise, *CEVI19* showed induced ectopic expression in viroid-infected leaves, while its constitutive expression in root and stem tissues was also maintained. The mode of expression of these newly isolated genes contrast to that observed for other defense-related genes which are induced de novo in infected plants and which do not show any constitutive expression in healthy plants during vegetative growth. The pattern of expression of one of such genes (encoding a basic PR-1 protein) is included in Figure 1 for comparison.

The association of ethylene with plant response to pathogens has been established in several pathosystems (Boller 1991) and is explained in part as a result of the induction of genes encoding ethylene biosynthetic enzymes (Knoester et al. 1995). The ethylene precursor 2-chloroethylphosphonic acid (ethephon) has been reported to stimulate accumulation of defense-related gene products when applied to fully expanded tomato leaves (Vera et al. 1993). It has been shown that local application of ethephon induces accumulation of mRNAs for some PR genes to even higher levels than those obtained by viroid infection (Domingo et al. 1994; Tornero et al. 1994). To establish whether this compound can reproduce the expression of the four viroid-inducible genes presently identified, tomato leaves (from 4- to 5-week-old plants) were treated with 1 mM ethephon solution and RNA samples were isolated from treated leaves at 60 h as described (Domingo et al. 1994). Results from these gel blots, as illustrated in Figure 1, revealed that transcripts corresponding to all clones accumulated, notably upon ethephon treatment. Consistent with our previous observations (Tornero et al. 1994), the gene encoding the basic PR-1 protein also showed a marked induction following ethephon treatment. Similar results were obtained in tobacco plants where genes for some PR proteins are expressed upon treatment with the ethylene precursor ethephon (Brederode et al. 1991; Linthorst et al. 1993). Ethephon may have additional ethylene-independent effects (Lawton et al. 1994) since in Arabidopsis ethephon enhances sensitivity to salicylic acid (SA), a signal molecule mediating defense gene activation and establishment of SAR. Thus the possibility exists that in tomato and tobacco, as well as in *Arabidopsis*, the effect of ethephon is mediated through activation of SA synthesis rather than by the action of ethylene per se.

The corresponding cDNA clones were sequenced and the deduced amino acid sequence compared with other previously described sequences. The nucleotide sequence of the cDNA clone CEVI16 was 1,160 nucleotides (Fig. 2). An open reading frame (ORF) extends from nucleotides 49 to 1045 of the cDNA sequence, encoding a preprotein of M_r 35914 (332 amino acids) with a putative signal sequence of 31 residues.

The mature polypeptide (301 amino acids, M_r 32320) was of a slightly cationic nature (pI, 7.56), and contains one glycosylation site (at position 215) (Fig. 2). A protein database search with the deduced amino acid sequence revealed extensive homology to many different plant peroxidases, including some from tomato (Botella et al. 1993; Vera et al. 1993). The highest identity score (80%) was obtained when compared to the secreted PNC2 peroxidase from peanut cell suspension cultures described by Buffard et al. (1990) (Fig. 3A). CEVI16 contains eight cysteine residues, which were located in similar positions in the primary sequences and three invariable histidine residues that have been inferred in the active-site structure of all peroxidases (Welinder 1992). Three domains homologous with other peroxidases can be noticed in CEVI16: The domain containing the histidine involved in the acid/base catalysis (at position 71); the domain containing the sequence VSCADIL; and the motif that includes the histidine residue involved in heme stabilization (at position 196 in CEVI16) (Welinder 1992).

CEVI16

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   1  GAATTCGGCACGAGAGAATTTTATCACAGTACGTACATAACACCAAATATGGAGTATTAC
   1                                                M  E  Y  Y
  61  AATTACAATTTAATCAACAAAATGGTTACAATTATTTTTATTCTGGTGCTAGTAATTGTT
   5  N  Y  N  L  I  N  K  M  V  T  I  I  F  I  L  V  L  V  I  V
 121  GATGTGACTATGGTATTCGGCCAAGGGACTCGTGTTGGATTTTATTCCAGTACGTGCCCA
  25  D  V  T  M  V  F  G  Q  G  T  R  V  G  F  Y  S  S  T  C  P
 181  AGGGCCGAATCCATAGTTCAATCAACGGTGAGGTCTCATTTTCAGTCTGATCCAACAGTG
  45  R  A  E  S  I  V  Q  S  T  V  R  S  H  F  Q  S  D  P  T  V
 241  GCACCAGGATTGTTGAGAATGCACTTTCATGATTGTTTCGTACAAGGTTGTGACGGTTCC
  65  A  P  G  L  L  R  M  H  F  H  D  C  F  V  Q  G  C  D  G  S
 301  ATCCTCATTTCTGGTACTGGCACTGAGAGAACAGCTCCTCCGAATTCCAATTTGAGAGGA
  85  I  L  I  S  G  T  G  T  E  R  T  A  P  P  N  S  N  L  R  G
 361  TTCGAGGTTATTGACGATGCTAAGCAGCAAATTGAAGCTGTTTGTCCTGGAGTTGTTTCA
 105  F  E  V  I  D  D  A  K  Q  Q  I  E  A  V  C  P  G  V  V  S
 421  TGTGCTGACATTCTTGCTCTTGCTGCTCGTGATTCCGTTCTCGTGACTAAAGGATTGACC
 125  C  A  D  I  L  A  L  A  A  R  D  S  V  L  V  T  K  G  L  T
 481  TGGTCTGTCCCCACGGGACGCACAGATGGACGAGTTTCAAGCGCATCAGACACATCTAAT
 145  W  S  V  P  T  G  R  T  D  G  R  V  S  S  A  S  D  T  S  N
 541  CTGCCAGGTTTTACTGAATCTGTTGCTGCTCAAAAGCAAAAGTTTGCTGCAAAGGGTCTC
 165  L  P  G  F  T  E  S  V  A  A  Q  K  Q  K  F  A  A  K  G  L
 601  AACACTCAAGATCTTGTCACCCTTGTTGGTGGGCACACAATTGGAACCTCAGCATGCCAA
 185  N  T  Q  D  L  V  T  L  V  G  G  H  T  I  G  T  S  A  C  Q
 661  TTCTTCAGCTACAGGCTATACAATTTCAACTCCACTGGTGGCCCTGACCCTTCAATAGAT
 205  F  F  S  Y  R  L  Y  N  F  N  S  T  G  G  P  D  P  S  I  D
 721  GCAACCTTTCTTTCTCAGCTTCAAGCATTATGTCCACAAAACGGAGATGGCTCGAAGCGT
 225  A  T  F  L  S  Q  L  Q  A  L  C  P  Q  N  G  D  G  S  K  R
 781  GTGGCACTGGACACTGGAAGCGTGAACAATTTTGACACCTCGTATTTCTCTAACTTGAGG
 245  V  A  L  D  T  G  S  V  N  N  F  D  T  S  Y  F  S  N  L  R
 841  AATGGTCGGGGAATTCTGGAATCAGACCAGATATTGTGGACCGATGCTTCGACCAAGGTG
 265  N  G  R  G  I  L  E  S  D  Q  I  L  W  T  D  A  S  T  K  V
 901  TTTGTCCAAAGGTATTTAGGCCTCAGGGGATTTCTTGGATTGAGATTCGGCTTAGAATTT
 285  F  V  Q  R  Y  L  G  L  R  G  F  L  G  L  R  F  G  L  E  F
 961  GGAAAGTCCATGGTTAAAATGAGCAATATTGAAGTTTTGACAGGGACTAATGGTGAAATT
 305  G  K  S  M  V  K  M  S  N  I  E  V  L  T  G  T  N  G  E  I
1021  CGTAAAGTTTGCTCTGCATTCAACTGATGCATATATGTATATACTAATTAGTTTCGTTCA
 325  R  K  V  C  S  A  F  N  *
1081  GTTAGGCAAGCTGCAAGATAAATTGTTAGTTATATCCAATATCAATAAATAAAAGCATTT
1141  ACCCACTGTTTTTTTTTTTT  1160
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CEVI19

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   1  CTAATTTCAGTGGGAGACTAAAAGGCTCTTCTCAATTTTGAGAATCAAAGAGGGAGGAAG
  61  ACGAAAATGGGAGGTGGTGGTAATATGTCTGCTATGACAAGTAAAAACGAACAGAAGAAA
   1        M  G  G  G  G  N  M  S  A  M  T  S  K  N  E  Q  K  K
 121  AATCCTCTCGAAAGAGTGCCATCTTCAAAGCCTCCTTTTACACTTGGTGATGTAAAGAAG
  19  N  P  L  E  R  V  P  S  S  K  P  P  F  T  L  G  D  V  K  K
 181  GCCATCCCTCCCCATTGCTTCGAACGATCTCTTGTAAAATCATTTTCGTTTCTTATCCAG
  39  A  I  P  P  H  C  F  E  R  S  L  V  K  S  F  S  F  L  I  Q
 241  GATCTGATACTCGTCTACATCTTTTATTACATCGCCAACACTTACATTCACCTCATTCCA
  59  D  L  I  L  V  Y  I  F  Y  Y  I  A  N  T  Y  I  H  L  I  P
 301  ACCCCATATAGGTATGTAGCATGGACCACTTATTGGATTGCTCAAGGTTGTGTTTGCACC
  79  T  P  Y  P  Y  V  A  W  T  T  Y  W  I  A  Q  G  C  V  C  T
 361  GGAATATGGGTCATTGGCCATGAATGTGCTCACCATGGCTTTAGTGATTACCAATGGGTA
  99  G  I  W  V  I  G  H  E  C  G  H  H  G  F  S  D  Y  Q  W  V
 421  GATGACATTGTTGGTCTTATCCTCCACTCAGCTCTTCTAACGCCATACTTTGCATGGAAA
 119  D  D  I  V  G  L  I  L  H  S  A  L  L  T  P  Y  F  A  W  K
 481  CATAGTCATCGTCGTCACCATGCAAACACAGGTTCCCTTGAGAATGATGAAATTTACATA
 139  H  S  H  R  R  H  H  A  N  T  G  S  L  E  N  D  E  I  Y  I
 541  CCAAGACTTAAGTCAAAACTAAGAGGCAAAAAATATGACCGTTTTGCATGTCATTATGAT
 159  P  R  L  K  S  K  L  R  G  K  K  Y  D  R  F  A  C  H  Y  D
 601  CCATATAGCCCAATATATTCAAATCGCGAAAGGCTACAAATCTACATTTCAGATGTAGGT
 179  P  Y  S  P  I  Y  S  N  R  E  R  L  Q  I  Y  I  S  D  V  G
 661  GTGATTGCAACAACTTATTTATTATATCGCGTTACTTTGACACAAGGGCTAGCTGGATTT
 199  V  I  A  T  T  Y  L  L  Y  R  V  T  L  T  Q  G  L  A  G  F
 721  ATAGTGTTGATCACTCTTATGCACCACACTCACTCTTCATTGCCACATTATGATTCAAGT
 219  I  V  L  I  T  L  M  H  H  T  H  S  S  L  P  H  Y  D  S  S
 781  GAGTGGGATCATCTAAGAGGAGCTTTAGCTACGGTAGATAGAGATTATGGTTTGTTAAAT
 239  E  W  D  H  L  R  G  A  L  A  T  V  D  R  D  Y  G  L  L  N
 841  AAAGTGTTCCACAATGTTACGGATACACATGTTTTGCATCATATATTCTCATATATATCA
 259  K  V  F  H  N  V  T  D  T  H  V  L  H  H  I  F  S  Y  I  S
 901  CATTACCATGCAATGGAGGCAAAAAAAGCTATCAAACCATTGTTGGGAGAATACTACAAA
 279  H  Y  H  A  M  E  A  K  K  A  I  K  P  L  L  G  E  Y  Y  K
 961  TATGATGATACACCAATTTTAAAGGCAATGTGGAGGGATACAAAGGAGTGTATCTTTGTG
 299  Y  D  D  T  P  I  L  K  A  M  W  R  D  T  K  E  C  I  F  V
1021  GAGAAAGATAAAGATAAAGGAGTTTATTGGTACAAAAACAAGCTTTGAAAAGTTCATCAA
 319  E  K  D  K  D  K  G  V  Y  W  Y  K  N  K  L  *
1081  GAAATAGTCATTTGGAATTGGTCAAATGTTAGTATTTGTATTTTGAATTCTATGTTTTTC
1141  TTGCATTGTACTAGTCAAGTGTTTGTCAAC  1170
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CEVI34

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   1  GACTCAAACTCCCCACATGGTGTTCGCTTACTGATTCAGGACTATCCATATGCTGTTGAT
   1  D  S  N  S  P  H  G  V  R  L  L  I  Q  D  Y  P  Y  A  V  D
  61  GGGTTGGAAATTTGGTCAGCAATCAAAAGTTGGGTAACAGAATATTGCAACTACTATTAC
  21  G  L  E  I  W  S  A  I  K  S  W  V  T  E  Y  C  N  Y  Y  Y
 121  AAATCAGATGACGCGGTACAGAAAGACGCTGAACTCCAAGCCTGGTGGAAGGAGCTCCGC
  41  K  S  D  D  A  V  Q  K  D  A  E  L  Q  A  W  W  K  E  L  R
 181  GAAGAAGGACATGGCGACAAGAAAGATGAGCCTTGGTGGCCTAAAATGCAAAGTGTGCAA
  61  E  E  G  H  G  D  K  K  D  E  P  W  W  P  K  M  Q  S  V  Q
 241  GAGCTTATAGATTCCTGCACCATCACTATATGGATAGCTTCAGCACTTCATGCAGCAGTT
  81  E  L  I  D  S  C  T  I  T  I  W  I  A  S  A  L  H  A  A  V
 301  AATTTTGGGCAATACCCTTATGCTGGTTATCTCCCTAATCGGCCTACATTAAGCCGGAAA
 101  N  F  G  Q  Y  P  Y  A  G  Y  L  P  N  R  P  T  L  S  R  K
 361  TTCATGCCAGAGCCAGGAAGTGCTGAGTATGAAGAGCTGAAGAGAAATCCAGATAATGTA
 121  F  M  P  E  P  G  S  A  E  Y  E  E  L  K  R  N  P  D  N  V
 421  TTCCTCAAAACAATCACTCCTCAGCTGCAGACATTGGTTGGAATTTCCCTTATAGAGCTC
 141  F  L  K  T  I  T  P  Q  L  Q  T  L  V  G  I  S  L  I  E  L
 481  TTGTCAAGGCATGCTTCGGATACCCTTTACCTCGGACAGAGGGACTCACCTGAATGGACA
 161  L  S  R  H  A  S  D  T  L  Y  L  G  Q  R  D  S  P  E  W  T
 541  AAGGATCAAGAACCACTTTCAGCTTTTGAGAGGTTCGGAAAGAAGTTGGGTGAGATCGAG
 181  K  D  Q  E  P  L  S  A  F  E  R  F  G  K  K  L  G  E  I  E
 601  GATCGAATTATTCAGATGAATGGTGATAATCAGAAATGGAAGAACAGGTCAGGGCCTGTT
 201  D  R  I  I  Q  M  N  G  D  N  Q  K  W  K  N  R  S  G  P  V
 661  AAAGTTCCATATACTTTCCTCTTTCCCACCAGTGAAGAGGGACTCACAGGCAAAGGAATC
 221  K  V  P  Y  T  F  L  F  P  T  S  E  E  G  L  T  G  K  G  I
 721  CCCAACAGTGTGTCTATATAGAACTTATTATTGTATCCCCTTGTTGTGCTTGTTTCTTCA
 241  P  N  S  V  S  I  *
 781  TATTAATTCCCAATAATATCACCAAATAAATTCTAGTTACAGTAGGGCAGTAATGTTGTT
 841  GGAGTTACTGTAA  853
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CEVI57

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   1  TAAATCTAAGTTAATTACGATAATTTATAAAGCATATCTACAATGGCTGTTTACAAAGTT
   1                                                M  A  V  Y  K  V
  61  AGTTTCCTTGCTCACCTACTTGTTCTTGGAATGTATCTACTAGTAAGCACGGTGGAACAC
   7  S  F  L  A  H  L  L  V  L  G  M  Y  L  L  V  S  T  V  E  H
 121  GCTAATGCTTGTACTAAAGAATGTGGTAATCTTGGCTATGGGATATGCCCAGGTTCAGAA
  27  A  N  A  C  T  K  E  C  G  N  L  G  Y  G  I  C  P  G  S  E
 181  GGAAGTCCAGAAAATCCAATATGTACCAATTGTTGCTCTGGCTATAAGGGTTGCAACTAT
  47  G  S  P  E  N  P  I  C  T  N  C  C  S  G  Y  K  G  C  N  Y
 241  TATTACGCTAATGGAACTTTTATTTGTGAAGGAACGTCTGATCCAAAAAATCCTAACATT
  67  Y  Y  A  N  G  T  F  I  C  E  G  T  S  D  P  K  N  P  N  I
 301  TGCCCCTCATATTGTGATCCACAAATTGCCTATTCAAAGTGTCCACGTTCAGAAGGAAAG
  87  C  P  S  Y  C  D  P  Q  I  A  Y  S  K  C  P  R  S  E  G  K
 361  ACGATAATCTATCCCACAGGATGTACGACGTGTTGCACTGGTTACAAGGGTTGCTACTAT
 107  T  I  I  Y  P  T  G  C  T  T  C  C  T  G  Y  K  G  C  Y  Y
 421  TTTGGTCAAGATGGAGAGTTTGTGTGTGAAGGAGAGAGTATTGAACCTAAGGGTTGTACT
 127  F  G  Q  D  G  E  F  V  C  E  G  E  S  I  E  P  K  G  C  T
 481  AAAGAATGTGACCCTAGAGTTGCTTATATGACTTGTCCATCTTCTGGATTGGCAAAACTT
 147  K  E  C  D  P  R  V  A  Y  M  T  C  P  S  S  G  L  A  K  L
 541  AATCAAGTTTGTGTTAATTGTTGTAGTGCAGGAGAGGGTTGCAAACTCTATGATAATGAT
 167  N  Q  V  C  V  N  C  C  S  A  G  E  G  C  K  L  Y  D  N  D
 601  GGATCTTTACTTTGTACTGGGGAGCCTCAAAGTATTTCCACAGCATAAGGAAGTTGGTTA
 187  G  S  L  L  C  T  G  E  P  Q  S  I  S  T  A  *
 661  TTTCTATTCAATGTTGTAGTTTATTAATGTATGAAATAAAAGAATGAATGGTGATATATG
 721  ATGCTATTTTATTAAAGTTCAAATTAAATAATTAAAGGTTTCAGTG  766
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Fig. 2. Nucleotide and deduced amino acid sequences of the tomato CEVI16, CEVI19, CEVI34, and CEVI57 cDNA clones. The predicted amino acid sequence is given in single-letter code. The predicted signal peptide in CEVI16 and CEVI57 is underlined. Stops codon are represented with asterisks. DNA sequence analysis was performed as described by Hattoni and Sakaki (1987).

Fig. 3. Alignment of the deduced amino acid sequences of the viroid-induced cDNA clones of tomato plants with those of other selected genes. **A,** Amino acid sequence comparison of the mature CEVI16 polypeptide with different plant peroxidases; PNC2 peroxidase from peanut cells (Buffard et al. 1990), Letpx2a peroxidase from tomato (Botella et al. 1993), CEVI1 peroxidase from tomato (Vera et al. 1993), Nsanper peroxidase from tobacco mesophyll protoplast (Criqui et al. 1992) and Atprxcb peroxidase gene from *Arabidopsis* (Intapruk et al. 1994). Conserved domains are underlined with a solid line, and conserved cystein and histidine residues are indicated with asterisk and triangles, respectively. **B,** Amino acid sequence comparison of CEVI19 with the *Arabidopsis* FAD2 desaturase (Okuley et al. 1994). Marked with solid lines are motifs conserved in different members of the desaturase family and which are also conserved in CEVI19. **C,** Amino acid sequence comparison of the derived CEVI34 polypeptide with different lipoxygenases from tomato fruits (LoxTom1, LoxTom2 (Ferrie et al. 1994)) and from *Arabidopsis* (LoxAth (Melan et al. 1993)). **D,** Sequence comparison of CEVI57 with different proteinase inhibitors of the class II described in tomato (Graham et al. 1985), potato (Thornburg et al. 1987) and tobacco (Baladin et al. 1995). The conserved catalytic motifs are boxed and designated as motifs I, II, and III, respectively. Conserved cystein residues are indicated with asterisks. Identical amino acids are indicated by a dash, and gaps (introduced to produce the best alignment) are indicated by dots. Sequence analyses were done using FASTA, MAP, and BESTFIT routines of the University of Wisconsin Genetic Computer Group package (Devereux et al. 1987).

The cDNA clone CEVI19 (1,170 base pairs) contained an ORF of 333 amino acids (Fig. 2) that could encode a protein of about 38,725 kDa and a pI of 9.1. Comparison of the predicted amino acid sequence with different databases revealed significant sequence similarity to the *Arabidopsis FAD2* gene encoding the oleate 12-desaturase (Okuley et al. 1994). The homology was maintained along the sequence (Fig. 3B), with 80% similarity and 64% identity observed between the two proteins. The hydropathic plots (data not shown) were nearly identical, with the exception that CEVI19 lacks the putative third transmembrane domain present in FAD2 and in other members of the desaturase family (Okuley et al. 1994). This structural difference could affect the topology of CEVI19 once inserted into the endoplasmic reticulum membranes. This lost membrane insertion domain coincides with a gap detected when comparing the amino acid sequence of CEVI19 and FAD2 (amino acids 166 to 199 of FAD2, see Fig. 3B). The tomato protein apparently has conserved the three histidine-rich motifs; the ExxH motif (residues 106-109), the HxxHH domain (position 268-272 of CEVI19) as well as the highly conserved WKYSHRRHH (positions 137-145) which are present among all desaturases and thought to be important in the active site of these enzymes, possibly as binding sites for the iron cofactor (Okuley et al. 1994). Comparison of the deduced amino acid sequence of CEVI19 with that of a range of plant desaturases indicated a degree of homology with CEVI19. For simplicity, only sequences from CEVI19 and FAD2 were used for the comparison shown in Figure 3.

The cDNA sequence of clone CEVI34 was 853 nucleotides (Fig. 2). RNA gel blot hybridization with this insert revealed a transcript of 2.8 kb (Fig. 1); this clone corresponds, therefore, to a very short copy of the endogenous RNA. In an attempt to obtain full-length cDNA copies, clone CEVI34 was used as a probe to rescreen the library. Several additional clones isolated did not extend further 5′ to the original insert in clone CEVI34. Additional attempts to obtain larger clones by PCR or 5′ RACE were also negative. Translation of the original CEVI34 clone predicted a protein sequence of 246 amino acids, followed by a 106-bp untranslated region. Comparison of the deduced amino acid sequence to the protein database revealed significant sequence homology to different members of the lipoxygenase (LOX) family (Fig. 3C). This comparison revealed that CEVI34 appears to constitute another member of the lipoxygenase family from tomato (Ferrie et al. 1994). Strong identity was also observed with the pathogen-induced LOX from *Arabidopsis* (Melan et al. 1993) and with LOX members from Pisum, soybean, and other plant species (data not shown).

Clone CEVI57 (766 nucleotides) (Fig. 2) shares extensive homology to prototypic class II proteinase inhibitors (PI-II) described in different members of the Solanaceae family (see Fig. 3D). The ORF encodes a preprotein of 201 amino acid residues that contains a highly hydrophobic, N-terminal region of 23 residues expected to function as a signal peptide similar to that of tomato or potato PI-II. However, the acidic mature polypeptide (178 amino acid residues, M_r 18867, pI, 4.5) encoded by *CEVI57* is considerably longer than the tomato and potato PI-II (Graham et al. 1985; Thornburg et al. 1987). Tomato and potato PI-II proteins are composed of two repeated domains involved in the inhibition of serine proteases (Greenblatt et al. 1989), while the CEVI57 protein is composed of a repeat of three of these domains. This is similar to the PI-II gene from tobacco, which is also induced in leaves by ethephon and by tobacco mosaic virus infection (Baladin et al. 1995). In all cases, the positions of the eight cysteine residues, involved in intramolecular folding (Greenblatt et al. 1989), were conserved. The putative domains of CEVI57 were compared with those of PI-II domains deduced from tomato, potato, and tobacco plants (Fig. 3D).

Many of the cytopathic changes occurring upon viroid infection are related to alteration in cell wall properties (i.e., irregular thickening of cell walls, increased cell-cell adhesion, inhibition of cell wall loosening, and extensibility, or resistance to protoplasting enzymes) (Wang et al. 1986; Semancik and Conejero 1987). These modifications are thought to make the affected cells more refractory to subsequent pathogen attacks. Secreted plant peroxidases have been implicated in several cell wall functions of potential importance in plant-pathogen interaction, such as in the polymerization of phenolic compounds (Grisebach 1981; Cottle and Kolattukudy 1982), wound-healing (Espelie et al. 1986), cross-linking of polysaccharides (Fry 1986), proline-rich proteins (Bradley et al. 1992), and pathogen defense (Hammerschmidt et al. 1982; Vance et al. 1980; Mohand and Kolattukudy 1990). Thus, the potential role on cell wall hardening and defense of the presently described *CEVI16* gene encoding a secreted peroxidase, as well as that of the distinct CEVI1 peroxidase recently described (Vera et al. 1993) can be entertained.

Resistance in many plant-pathogen interactions is accompanied by the rapid deployment of a multicomponent defense response (Dixon et al. 1994) which can be elicited by different chemical agents, and thus points to commonality of concerted signal transduction pathways operating in plants during different pathogenic situations. Jasmonic acid (JA) is recognized as one of the intracellular second messengers mediating a variety of cell responses, some of which are related to defense such as the systemin-mediated wound induction of proteinase inhibitor genes (Farmer and Ryan 1992), the elicitor activation of phytoalexin accumulation, or the induction of genes of the phenylpropanoid pathway (Creelman et al. 1992; Gundlach et al. 1992). In plants, jasmonic acid is synthesized by the lipoxygenase-mediated oxidation of polyunsaturated fatty acids like linoleic and linolenic acid (Vick and Zimmerman 1984). In contrast, defense-related stimulation of lipoxygenase gene expression and enzyme activity has been reported in a number of cases (Koch et al. 1992; Melan et al. 1993; Wanner et al. 1993; Peever and Higgins 1989). It thus seems that lipoxygenase induction, and in turn synthesis of JA, is likely to play an important role in the induction of the defense status of the plant. The identification that *CEVI34* encodes a putative member of the lipoxygenase family from tomato, suggests that JA is one of the signal molecules participating in the multicomponent signaling processes activated by viroid infection. The striking correlation between the mode of expression during normal development and also during pathogenesis of the *CEVI34* and *CEVI57* genes, the later encoding a novel proteinase inhibitor gene of the class II, parallels the mode of induction of other proteinase inhibitors regulated by JA. According to the model of Farmer and Ryan (1992), linolenic acid, a fatty acid synthesized by the FAD2 desaturase (Okuley et al. 1994), will be released from membranes in response to elicitation and converted to fatty acid hydroperoxides by the

action of a lipoxygenase. These lipoxygenase-derived fatty acid hydroperoxides are the precursors of jasmonic acid, which in turn will activate plant defenses. The observed activation of the *CEVI34* and *CEVI57* genes presently described suggests that a similar signal transduction pathway may be continuously activated by the viroid systemic infection. A cellular consequence that could be derived from this activation cascade is that linolenic acid pools would decrease in the membranes of affected cells. This would affect the physicochemical parameters of plasma membrane, and eventually, might lead to a cell death process which occurs in many incompatible plant-pathogen interactions (Tzeng and De Vay 1993). To generate a continuous supply of unsaturated lipids to counteract these deleterious effects to the the plasma membrane in the compatible viroid-host interaction, the plant must have developed complementary responses to cope with the imposed stress. It is thus conceivable that expression of genes related with synthesis of unsaturated fatty acids are to be altered in this pathogenic context in order to counteract deleterious effects. Consistent with this, the observation that the *CEVI19* gene encodes a homolog of the *Arabidopsis FAD2* gene, responsible for production of polyunsaturated lipids, and in particular linolenic acid, favors one possible explanation of why a gene encoding a desaturase-like enzyme is also activated following viroid infection. However, alternative roles for the defense-related genes presently described, not necessarily related to the above-mentioned scenario, cannot be disregarded. Such could be the case for the desaturase-like CEVI19 and the CEVI16 peroxidase, which could be involved in the biosynthesis of cutin and/or suberin which are critical plant defense barriers to pathogen ingress. Definitive proof of the function of the genes described in this paper must await assays with the proteins they encode as well as interpretation of their function in transgenic plants.

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