Indian bunchy top disease of tomato plants is caused by a distinct strain of citrus exocortis viroid


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A viroid has been isolated from tomato plants affected by Indian bunchy top disease of tomato (Lycopersicon esculentum Mill.). In dot blot hybridization assays with 32P-labelled cRNA probes specific for the detection of various viroids, the Indian viroid was shown to be most closely related to the citrus exocortis viroid (CEVd). Sequence determination showed that the viroid consists of 372 nucleotides and confirmed its close relationship with CEVd. The viroid, for which we propose the acronym CEVd-t, differs from the Australian CEVd strains A and B by 36 and 47 nucleotides, respectively, and from the Spanish grapevine isolate by 52 changes. A phylogenetic analysis confirmed the closest relationship with CEVd in all structural domains, except the pathogenicity and left-terminal domains, which are closely related to the corresponding domains of the potato spindle tuber and tomato apical stunt viroids, respectively.

Introduction

A tomato (Lycopersicon esculentum Mill.) disease, characterized by extensive apical proliferation and stunting, epinasty, various leaf distortions and veinal necrosis, was observed during a survey of tomato cultivation in the Pune region of Maharashtra, India in 1979. Pandey & Summanwar (1982) noticed that the syndrome resembled that of the tomato bunchy top disease described by McClean (1931, 1935) from South Africa and by Walter (1981) from West Africa.

Because the latter disease has been shown to be caused by a viroid, the tomato apical stunt viroid (TASVd), we hypothesized that a viroid might also be responsible for the Indian disease. We report here that the viroid in question is not, as was originally suspected, a strain of TASVd (Candresse et al., 1987; Kiefer et al., 1987), but is a distinct strain of the citrus exocortis viroid (CEVd) (Gross et al., 1982).

Methods

Isolation and propagation. The infectious agent was isolated from a single leaflet of an affected tomato plant (cv. Pusa Ruby). The leaflet was macerated in 0.05 M-K2HPO4 and mechanically inoculated to tomato plants (cv. Rutgers) at the cotyledonary stage. The inoculated plants were kept in an insect-proof greenhouse under quarantine at 28 °C with 18 h daylight. Systemically infected leaves showing typical symptoms of the disease were harvested 15 to 20 days post-inoculation (p.i.) and stored frozen at −20 °C. In addition, TASVd and tomato planta macho viroid (TPMVd) (Kiefer et al., 1987), CEVd (Visvader & Symons, 1985), citrus B viroid (CBVd; a strain of hop stunt viroid, HSVd) (Diener et al., 1988), chrysanthemum stunt viroid (CSVd) (Beltsville strain), and the mild and intermediate (Beltsville) strains of potato spindle tuber virus (PSTVd) were propagated in Rutgers tomato and the infected tissue was treated similarly.

Infectivity assays. Rutgers tomato plants at the cotyledonary stage were mechanically inoculated by gently rubbing a spatula, previously dipped into inoculum, over carborundum (600-mesh)-dusted cotyledons, followed immediately by thorough rinsing with distilled water.

Nucleic acid extraction. Tomato leaves were ground with a mortar and pestle to a powder in liquid nitrogen and nucleic acids were extracted by the direct phenol method (Diener, 1979). Briefly, leaf powder was homogenized with 1 ml/g tissue of 1 M-K2HPO4, pH 8.2 containing 1% ascorbic acid, 0.1 % sodium diethyl-dithiocarbamic acid and 2% SDS, and an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 10% of a chloroform-isopropanol mixture (25:1, v/v). The homogenate was concentrated in a Sorvall refrigerated centrifuge at 5000 g for 10 min. Total nucleic acids were then precipitated from the aqueous phase with 2 vol. of ethanol. Polysaccharides were removed by adding equal volumes of 2.5 M-K2HPO4, pH 8.0 and ethylene glycol monoethyl ether to the water-suspended nucleic acids.

After addition of 0.25 vol. of 1% cetetyltrimethylammmonium bromide to the separated upper phase from the previous step, followed by overnight storage at −20 °C, nucleic acids were repelleted by centrifugation at 7700 g for 20 min. The pellet was washed three times...
with 70% ethanol/0.1 M-sodium acetate and was finally resuspended in 10 mM-Tris-HCl pH 7.5, 2 mM-MgCl₂. The preparation was then treated with deoxyribonuclease (20 μg/ml), followed by phenol/chloroform extraction and precipitation. The preparation was then resuspended in distilled water and incubated in 2 M-LiCl as described previously (Diener, 1979).

PAGE. Return-gel PAGE was performed as described by Singh & Boucher (1987) with modifications. Approximately 20 μg of nucleic acid sample was electrophoresed through a 7.5% polyacrylamide slab gel (39:1 acrylamide:bisacrylamide) (15 x 16 x 0.12 cm) in 1 x TBE buffer (89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA) at 46 mA and 20 °C until the xylene cyanol was at the bottom of the gel. The polarity was reversed, the buffer in the chambers was replaced with a 1:8 dilution of the 1 x TBE buffer (70 °C in the top chamber, 100 °C in the lower chamber) and the second electrophoresis (46 mA) was performed at 70 °C with the aid of a heated plate. Electrophoresis was stopped after 1.5 h.

For further purification of the viroid, bidirectional electrophoresis was performed according to the method of Schumacher et al. (1983). Nucleic acids were detected by staining with either ethidium bromide or silver nitrate (Sammons et al., 1981).

Nucleic acid hybridization. Viroid-specific 32P-labelled cDNA and cRNA probes were prepared as described (Diener et al., 1988) and dot blot hybridization assays (Owens & Diener, 1981) were performed using probes specific for the detection of PSTVd, HSVd, TASVd, TPMVd and CEVd. Samples of nucleic acids were diluted fivefold in water and spotted onto nitrocellulose membranes which had been presoaked in 20 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate). The membranes were hybridized with cRNA probes and treated twice with ribonuclease as described (Diener et al., 1988).

PCR amplification, cDNA cloning and sequence analysis. The strategy used to amplify viroid cDNAs synthesized from low M₉ RNA templates has been described (Owens et al., 1990). Aliquots of randomly primed viroid cDNAs were amplified using two sets of primers, Had 3 (5' CTCCAGGTTTCCCCGGG 3') and RAO-2 (5' GCGGATCCGGTGGAAACAACTGAAGC 3') for the left half of the viroid and Had 4 (5' AGGGCTAAACACCCTCGCCC 3') and RAO-14 (5' AGGGATCCCCGGGGAAACC 3') for the right half of the viroid. Following PCR amplification, the double-stranded cDNAs were digested with BamHI and HindIII and inserted into the pUC9 plasmid which had been digested with BamHI and HindIII using previously described protocols (Maniatis et al., 1982). Recombinant plasmids were grown in Escherichia coli strain JM83 for further analysis (Maniatis et al., 1982). Selected cDNA clones were sequenced using the TaqTrack sequencing system for double-stranded plasmid DNA (Promega). The most stable secondary structure of the RNA was determined by the computer program of Zuker (1989).

Phylogenetic analysis. To assess possible evolutionary relationships, the nucleotide sequence of the Indian viroid was added to a previously prepared databank of aligned viroid and viroid-like RNA sequences (Diener, 1989), and phylogenetic relationships were estimated by the DNAPARS program included in the PHYLIP Phylogeny Inference Package (version 3.1) (Felsenstein, 1983).

Results

Detection of viroid-like RNA by PAGE

An RNA band in the viroid region was consistently present in diseased tomato plants when extracts were electrophoresed in return-gel PAGE (Fig. 1, lane 1 and lanes 3 to 10). No band was detected in this region with identical preparations from healthy plants (Fig. 1, lane 2). The suspected Indian viroid (lane 7) had a unique electrophoretic mobility as compared with samples of PSTVd (lanes 3 and 4), TASVd (lanes 1, 5 and 10), CEVd (lanes 6 and 8) and CBVd (lane 9) run in the same gel.

Infectivity of disease-specific RNA

Gel bands containing the presumed viroid were excised, and the RNA was eluted and assayed for infectivity on tomato plants. Symptoms typical of the Indian bunchy top disease developed in 45% of the inoculated plants 20 days p.i. No symptoms developed in plant that were either buffer-inoculated or inoculated with material eluted from corresponding regions of lanes in which extracts from healthy plants were electrophoresed. These infectivity experiments proved that the disease-associated RNA is a viroid and that it is the causative agent of Indian bunchy top disease.

Similarities with other viroids

In dot blot hybridization tests with cRNAs specific to PSTVd, TPMVd, TASVd, CEVd and HSVd, all probes with the exception of the HSVd cRNA reacted with the Indian viroid before treatment with RNase (Fig. 2a). After one treatment with RNase (Fig. 2b), strong signals were present only with TPMVd, TASVd and CEVd.
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cRNAs; and after another incubation with RNase (Fig. 2c) the strongest signal remaining was obtained with CEVd cRNA, as well as a weaker one with TASVd cRNA.

Sequence determination and presumed secondary structure

As shown in Fig. 3, the viroid consists of 372 nucleotides, composed of 72 A, 109 C, 113 G and 78 U residues, thus resulting in a G+C content of 59-7% and a G+C/A+U ratio of 1.48. The most thermodynamically favourable secondary structure consists of a highly base-paired, quasi double-stranded rod, in which short helical regions alternate with internal and bulge loops (Fig. 3).

Phylogenetic relationships

Analysis of the five structural domains of the Indian viroid sequence and comparison with those of other viroids and viroid-like satellite RNAs using the DNAPARS program (Felsenstein, 1983) gave the results shown in Table 1. The central, variable and right-terminal domains are most closely related to those of CEVd, whereas the pathogenicity and left-terminal domains are most closely related to those of PSTVd and TASVd, respectively.

Table 1. Nearest neighbour analysis of CEVd-t*

<table>
<thead>
<tr>
<th>Domain†</th>
<th>CEVd-</th>
<th>PSTVd-</th>
<th>TASVd-</th>
<th>TPMVd</th>
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<tbody>
<tr>
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<td>A</td>
<td>B</td>
<td>g</td>
<td>I</td>
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<tr>
<td>Pathogenicity</td>
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<td>25</td>
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<td>13</td>
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<td>3</td>
<td>51</td>
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<tr>
<td>Right-terminal</td>
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<td>5</td>
<td>11</td>
<td>43</td>
</tr>
</tbody>
</table>

* Number of nucleotide changes from CEVd-t in the most parsimonious phylogenetic trees was measured.
† CEVd: A, Australian strain A (Visvader & Symons, 1985); B, Australian strain B (Visvader & Symons, 1985); g, Spanish grapevine strain (García-Arenal et al., 1987). PSTVd: intermediate strain (Schnöller et al., 1985); M, mild strain (Schnöller et al., 1985); S, severe strain (Schnöller et al., 1985). TASVd: A, African strain (Kiefer et al., 1983); I, Indonesian strain (Candresse et al., 1987).
‡ The boundaries of the five structural domains in CEVd-t are: nucleotides 326 to 372/1 to 46, left-terminal domain; nucleotides 75 to 294 and 244 to 293, central conserved region; nucleotides 122 to 150 and 215 to 243, variable region; nucleotides 151 to 214, right-terminal domain.
Discussion

Our results show that Indian bunchy top disease of tomatoes is caused by a viroid. Contrary to expectations, however, this viroid is not a strain of TASVd or TPMVd, but is instead a distinct strain of CEVd. Although the tomato is an experimental host of CEVd, this viroid has not previously been associated with a disease of cultivated tomatoes. The close relationship of the viroid with CEVd was first indicated in dot blot hybridization assays with cRNAs specific to a number of common viroids and sequence analysis confirmed that the viroid is a distinct strain of CEVd. We propose the acronym CEVd-t for the viroid. Although both TASVd (Walter, 1981; Candresse et al., 1987) and TPMVd (Galindo et al., 1982) are responsible for naturally occurring tomato diseases, Indian bunchy top disease is the first known tomato viroid disease caused by a strain of CEVd.

The question arises whether the tomato bunchy top disease described from South Africa (McClean, 1931, 1935) is caused by a viroid and, if so, by a strain of TASVd, TPMVd, CEVd or by yet another viroid. Unfortunately, identity of the agent cannot be determined because, as discussed elsewhere (Diener, 1987), no bunchy top diseased tomato tissue could be obtained from South Africa.

As shown in Fig. 3, CEVd-t is most closely related to Australian type strain A (Visvader & Symons, 1985), differing by 36 changes (29 nucleotide substitutions, three insertions and four deletions). Somewhat more distant is Australian type strain B (Visvader & Symons, 1985), from which it differs by 47 changes (38 substitutions, four insertions and four deletions). Still more distantly related is the Spanish grapevine strain of CEVd (CEVd-g) (Garcia-Arenal et al., 1987) with 53 changes (46 substitutions, two insertions and five deletions).

As is the case with CEVd-g, the nucleotide sequence of CEVd-t differs more from those of type strains A or B than the latter do between themselves. As has been suggested by Garcia-Arenal et al. (1987), this relative remoteness of CEVd-g and CEVd-t from CEVd strains isolated from citrus may be a consequence of their different host origin, i.e. grapevines in the case of CEVd-g and tomatoes in the case of CEVd-t. The closer similarity of CEVd-t to CEVd strains that cause severe symptoms on tomatoes (type A) than to mild strains (type B) is consistent with the severe symptoms produced by CEVd-t in its natural host, the tomato.

Changes that transform CEVd-A into CEVd-t are present primarily in the left-terminal, pathogenicity and variable domains, whereas the central domain and the right-terminal region undergo essentially no changes (Fig. 3). CEVd-t also resembles CEVd-g in this respect (Garcia-Arenal et al., 1987), except that with the latter, the right-terminal region, as well as the left one, differs significantly from that of CEVd-A.

Nucleotide changes between CEVd strains leading to modifications in the presumed secondary structures of the pathogenicity domain have been implicated in the modification of symptom expression on tomatoes (Visvader & Symons, 1985, 1986); and Garcia-Arenal et al. (1987) have pointed out that most of the changes that lead to the different secondary structure of the pathogenicity domain of mild strains (when compared to severe strains) are located outside of a central core, which is strictly conserved in all severe strains. This central core consists of an A₆₆₆ sequence (mostly in an internal loop) and the adjoining base-paired stems. Examination of the central core of CEVd-t shows that its secondary structure is identical to that of all severe strains, except that the eight base helical region to the right of the oligo(A) sequence present in all severe CEVd strains is interrupted by an insertion of five nucleotides (leading to an internal loop of three U residues and an additional A-U base pair). Lowering of the thermodynamic stability of this local region does not necessarily affect the severity of symptom expression. This is verified further by the fact that the central core of the pathogenicity domain present in all severe strains of CEVd (except CEVd-t) also occurs in all strains of PSTVd, regardless of whether they produce mild, intermediate or severe symptoms in tomatoes (Schnölzer et al., 1985).

Phylogenetic analysis was performed with each structural domain individually because, with viroids, evidence of chimeras suggests that corresponding domains of different viroids are exchangeable. This is most clearly shown by the nucleotide sequence of the Columnea latent viroid which consists predominantly of sequences previously shown to be present in other viroids (Hammond et al., 1989) and by the sequence of the Australian grapevine viroid (Rezaian, 1990), which similarly suggests that this viroid is the product of extensive RNA recombination.

Phylogenetic analysis by the parsimony method (Felsenstein, 1983) revealed, as expected from direct sequence comparisons, close relationships between most domains of CEVd-t and CEVd. Interestingly, however, the pathogenicity and left-terminal domains had their closest phylogenetic relationships with the corresponding domains of PSTVd and TASVd, respectively. CEVd-t, therefore, may serve as a ‘missing link’ between those viroids which cause symptoms in tomatoes. Conceivably, close similarity of these domains with those of other viroids causing naturally occurring tomato diseases may be a consequence of host adaptation of the Indian strain of CEVd.
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References


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