Tomato chlorotic dwarf viroid: an evolutionary link in the origin of pospiviroids

Rudra P. Singh,1 Xianzhou Nie1 and Mathuresh Singh2

1 Agriculture and Agri-Food Canada, Potato Research Centre, PO Box 20280, Fredericton, New Brunswick, Canada E3B 4Z7
2 Agricultural Certification Services, NB Potato Agency, 245 Hilton Road, Unit 25, Fredericton, New Brunswick, Canada E3B 5N6

Over 40 isolates of potato spindle tuber viroid (PSTVd) have been reported from potato, other Solanum species and greenhouse tomato. These isolates have sequence similarities in the range 95–99%. A viroid which caused chlorotic leaves and severe dwarfing of plants in greenhouse tomato crops was detected. The viroid was found to hybridize readily with PSTVd probes. It migrated faster than PSTVd in return-polyacrylamide gel electrophoresis and was not amplified in RT–PCR by a primer pair based on the lower strand of the central conserved region of PSTVd. Nucleotide sequencing of the viroid indicated that it is a circular RNA of 360 nt, with less than 90% sequence similarities with PSTVd isolates. The Variable domain (V) has less than 60% and the Terminal Right domain less than 90% sequence similarity, while the remainder of the molecule has greater than 97% similarity with PSTVd. Because of its less-than 90% sequence similarities, unique V domain, lack of seed-transmission and lack of cross-protection by PSTVd, the viroid from tomato is proposed to be a distinct viroid species (tomato chlorotic dwarf viroid; TCDVd) which also differs from two viroids infecting tomato in nature. TCDVd may be an evolutionary link in the development of crop viroids, with Mexican papita viroid as the ancestral viroid.

Introduction

The low-molecular-mass plant pathogens, the viroids, are 246–399 nt circular RNA molecules (Singh, 1998). Over 25 plant maladies have now been ascribed to viroids in agricultural, horticultural and ornamental plants (Diener, 1987; Singh & Dhar, 1998). Nucleotide sequences of almost all viroids are known along with their many sequence variants. In some cases, these sequences have been correlated with symptom severity and have been assigned to viroid strains (Visvader & Symons, 1985; Schönzer et al., 1985; Herold et al., 1992; Gora et al., 1994). Isolates with higher than 90% sequence similarities are generally considered as variants of a particular viroid, while a viroid is generally considered a distinct species if similarities are less than 90% (Flores et al., 1998).

Potato spindle tuber viroid (PSTVd), with its numerous, well-characterized strains and isolates, was the first viroid to be discovered (Diener, 1971; Singh & Clark, 1971), structurally characterized (Sänger et al., 1976) and sequenced (Gross et al., 1978). It is the type species of the genus Pospiviroid (Flores et al., 1998). About 40 PSTVd variant sequences are found in the GenBank and EMBL nucleotide databases. The potato (Solanum tuberosum) isolates represent the natural source of infection in the field (Schnölzer et al., 1985; Owens et al., 1992; Herold et al., 1992; Lakshman & Tavantzis, 1993; Singh et al., 1993; Gora et al., 1994; Gora-Sochacka et al., 1997). Other isolates are in vitro-generated PSTVd mutants (Hammond, 1992, 1994; Lakshman & Tavantzis, 1992; Qu et al., 1993; Wassenegger et al., 1994, 1996; Owens et al., 1995; Hu et al., 1996). Except for one isolate of 341 nt (Wassenegger et al., 1994), such isolates are very similar in size and vary by 1–7 nt from the prototype strain (Gross et al., 1978). Naturally occurring PSTVd isolates from greenhouse tomato (Lycopersicon esculentum) and Solanum species (including S. muricatum) have also been reported (Puchta et al., 1990; Owens et al., 1992; Behjatnia et al., 1996; Shamloul et al., 1997). These isolates have sequence similarity of 95–98% with PSTVd. Greenhouse tomato and pepino (S. muricatum) isolates are 3–5 nt shorter than the 359 nt PSTVd. It has been assumed that accidental transfers of PSTVd from symptomless pepino could have
caused symptoms in greenhouse tomatoes, since pepino seed is generally infected with PSTVd (Puchta et al., 1990).

In Canada, PSTVd has been eradicated (Singh, 1988) and has not been detected in potato fields since 1980. Thus, when approximately 2000 greenhouse tomato seedlings of cv. Trust, grown from seed imported from The Netherlands, suddenly developed chlorotic leaves and grew to become severely dwarfed and bunched plants, i.e. symptomology typical of PSTVd in tomato (Singh & O’Brien, 1970), a viroid aetiology was suspected. We report here identification of a viroid from these plants, designated tomato chlorotic dwarf viroid (TCDVd), which has sequence similarities of 86–88% with PSTVd potato isolates and 85–89% with other (greenhouse tomato, pepino, Solanum species) isolates. Considering that Mexican papita viroid (MPVd) (Martinez-Soriano et al., 1996), which has 78–80% sequence identity to PSTVd, has been proposed as an ancestor of crop viroids, TCDVd may represent an evolutionary link between MPVd and PSTVd.

**Methods**

**Disease material, transmission, host-range and cross-protection.** Diseased plants of tomato cv. Trust with severely reduced leaves and fruits were received from a greenhouse tomato producer (Carman, Manitoba, Canada). A few symptomatic leaves were ground in buffer (50 mM glycine, 30 mM phosphate, pH 9.2) and sap was manually inoculated to tomato cv. Sheyenne and Trust by rubbing onto leaves dusted with 350 mesh Carborundum. Graft inoculation was performed by inserting stem scions from diseased tomato plants into a downward cut in the stem of stock plants, and the two were bound together with a Parafilm strip. These plants served as the disease source from which to inoculate other plants by graft or manual inoculation. Twelve commonly used indicator plants and ten potato cultivars were used in host-range studies. Seeds from infected indicator plants and two potato cultivars were used for seed transmission tests. R-PAGE (see below) was used to confirm the presence of TCDVd in subsequent plant inoculations. Cross-protection (Khoury et al., 1988) was performed by pre-inoculating with PSTVd, followed by challenge inoculation with viroid from tomato.

**Viroid isolation, RT–PCR, cloning and sequencing.** Total leaf nucleic acids were extracted and return-polyacrylamide gel electrophoresis (R-PAGE) was carried out (Singh, 1991). About 1.5 µg (6 µl) of nucleic acid was loaded on a 5% gel and the first cycle carried out under non-denaturing conditions in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA; pH 8.3) at 25 °C. The reverse cycle was carried out under denaturing conditions (1.8 diluted buffer, at 71 °C). RNA bands were visualized by silver staining.

An RT–PCR for preliminary characterization of viroids was carried out using three primer pairs, based on the sequences of PSTVd isolates. Primers 2A (5’ TGTTTCCACCGTATGACG 3’; complementary to nt 254–273) and 1S (5’ ACTCTGTTGTTCTTGGGTC 3’; identical to nt 10–29) were designed for use with isolate FM (Herold et al., 1992). P1 (complementary to nt 256–273) and P2 (identical to nt 274–295) were from PSTVd (Gross et al., 1978); P3 (complementary to nt 86–93) and P4 (identical to nt 87–110), for use with the Darwin isolate of PSTVd, were the primers used by Behjatnia et al. (1996).

For purification of viroid, total leaf nucleic acids from TCDVd-infected Nicandra, potato, Scopolia and tomato were separated in R-PAGE and unstained viroid bands (Singh et al., 1988) were cut out and incubated overnight at 37 °C in eluting buffer (0.5 M ammonium acetate, 0.1% SDS, 10 mM EDTA; pH 8.0). Eluted viroid RNA was precipitated with ethanol and suspended in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). cDNA was synthesized and amplified with uracil-containing primers for subsequent cloning as in Rashtchian et al. (1992). Briefly, CUACA-CUACUA was added onto primer P3 and CAUCAUCAUCA onto P4 at the 5’ end (Singh & Singh, 1995). Amplification was carried out for 35 cycles (1 min each of denaturation at 94 °C, annealing at 55 °C and extension at 72 °C, with a final extension for 7 min). PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Bands of approximately 400 bp were excised, extracted with TE, purified with Glass MAX spin cartridges (Gibco BRL) and then cloned into competent Escherichia coli DH5α using the clone Amp 1 system (Gibco BRL). Viroid inserts were confirmed by PCR and agarose gel electrophoresis.

Two viroid clones, each of which infected Nicandra, potato, Scopolia and tomato, were initially sequenced in both directions by the dyeoxy chain-termination method (Sanger et al., 1977) using modified T7 DNA polymerase (Sequenase version 2.0, US Biochemical). Later, the sequencing was done using a PE/ABI 377 DNA sequencer and the PE/ABI ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems). In order to confirm the sequence in the T3+P4 priming region, a second pair of primers (P5, P6) was designed. P5 is complementary to nt 342–360 and P6 is identical to nt 1–20 of TCDVd (this study); these primers were synthesized with uracil NTPs, and amplification products were cloned and sequenced as before. Again, eight clones (two from each host) were sequenced as above. Sequence analysis by pair-wise comparison and multiple alignment, as well as calculation of secondary structures, were carried out using the Genetics Computer Group suite of programs, version 8.1 (University of Wisconsin, Madison, WI, USA).

**Results and Discussion**

**Symptoms, host-range, seed transmission and cross protection**

Sheyenne tomato plants grafted or manually inoculated with diseased samples developed symptoms. The symptoms included leaf chlorosis, vein and petiolar necrosis, reduction of leaf size and overall bunchiness and dwarfing of the plant, a symptomology mimicking that of PSTVd in this host (Singh & O’Brien, 1970). A more severe but similar symptomology was observed in tomato cv. Trust. Total nucleic acids extracted from original diseased Trust tomato and further multiplied in Sheyenne tomato plants displayed a band migrating slightly faster than PSTVd FM in R-PAGE (Fig. 1, lane 15). This viroid band was tentatively designated tomato chlorotic dwarf viroid (TCDVd).

Plants of Nicandra physaloides, Nicotiana debneyi, Nicotiana glutinosa, Physalis angulata, P. floridana, Scopolia sinensis, Solanum demissum, S. tuberosum cv. Alpha, Atlantic, Chieftain, Chipeta, Niska, Red Pontiac, Russel Burbank, Superior and Yukon Gold were found to be susceptible to TCDVd. Symptoms of leaf and petiole necrosis were observed in N. physaloides and S. sinensis plants, while severe dwarfing and upright growth of all potato cultivars were the dominating symptoms. Variegated flower petals (colour break) were characteristically observed in N. physaloides and N. glutinosa. Severe cracking of potato tubers without noticeable elongation of the tuber size was also
Tomato chlorotic dwarf viroid

observed with TCDVd infection. Plants of *Datura metel*, *Gomphrena globosa* and *Nicotiana tabacum* were not susceptible.

PSTVd is seed-transmitted and can persist in true potato seed for over 20 years (Singh et al., 1988, 1991). The possibility that TCDVd is seed-borne was tested by R-PAGE using 100 seeds of each indicator plant and two potato cultivars: a total of 700 seeds and 400 seedlings of *N. physaloides*, *N. debneyi*, *P. angulata*, *P. floridana*, tomato and potato cvs Atlantic and Superior were tested for seed-borne infection of TCDVd. No infection was detected by R-PAGE either in the seeds or plants generated from seeds. In parallel experiments, PSTVd infection of 7-year-old true potato seed was routinely detected by R-PAGE.

Because the differential migration pattern of TCDVd and PSTVd in R-PAGE allows the two species to be differentiated, cross-protection for viroid replication could be tested (Khoury et al., 1988). Ten tomato plants pre-infected with PSTVd FM (Herold et al., 1992) and challenged with TCDVd supported replication of both viroids (Fig. 1, lanes 6–9) similar to the singly infected plants (Fig. 1, lanes 2–5 and 10–13). The mild symptoms of PSTVd FM were replaced with the severe symptoms characteristic of TCDVd in doubly infected plants. We concluded that there was no interference by PSTVd in the multiplication and symptom expression of TCDVd in doubly infected plants.

RT–PCR, cloning and sequence determination

Primer pair 2A + 1S amplified two bands of different sizes from TCDVd and PSTVd. The main PSTVd band had a counterpart of similar mobility from the TCDVd-infected sources (Fig. 2, upper panel). P1 + P2 amplified PSTVd but not TCDVd (Fig. 2, middle panel) and P3 + P4 amplified a single band in both viroid infections (Fig. 2, lower panel). These observations indicated that TCDVd might differ in sequence in the lower strand of the central conserved region, a difference which the primers P1 + P2 were designed to detect. Since the P3 + P4 primer pair, based upon the upper strand of the central conserved region (CCR), gave a full-length PCR product for both PSTVd and TCDVd, we suspected that there would be similarities of sequences in the upper strand of the CCR.

Analysis of 16 clones sequenced in both directions showed that TCDVd consists of 360 nt (Fig. 3). There was minimal variation between sequences of clones and from different hosts. Of the 16 clones only two (one each from *N. physaloides* and *S. sinensis*) showed one nucleotide change, in *N. physaloides* U₁₄₅ → C, and in *S. sinensis* U₂₁₁ → A. The DNA inserts were infectious in tomato and *S. sinensis* plants when removed from plasmids by BamHI excision, indicating the accuracy of the predominant nucleotide sequence.

TCDVd assumes a thermodynamically stable rod-like secondary structure similar to other members of the family Pospiviroidae (Fig. 3). Sequence comparison showed that TCDVd is more closely related to PSTVd isolates than to any other viroids. TCDVd has nucleotide sequence similarities of 86-5% with PSTVd ‘type’ strain (Gross et al., 1978), 86-8% with PSTVd-Darwin (Behjatnia et al., 1996), 89-6% with
Fig. 3. Primary and secondary structure of TCDVd. The approximate location of various domains are indicated. TL, Terminal Left; P, Pathogenicity; C, Central Conserved Region; V, Variable; and TR, Terminal Right.

Fig. 4. GrowTree phylogram of members of the genus Pospiviroid, indicating the distinct position of the TCDVd in comparison to PSTVd. Distances are estimated number of substitutions per 100 bases. MPVd, Mexican papita viroid; TPMVd, tomato planta macho viroid; CEVd, Citrus exocortis viroid; TASVd, tomato apical stunt viroid; CSVd, Chrysanthemum stunt viroid; CLVd, Columnea latent viroid; and IrVd, Iresine viroid.

PSTVd-N (Puchta et al., 1990), 85.5% with the published 354 nt PSTVd-Chile sequence (Shamloul et al., 1997) and 80.3% with MPVd (Martinez-Soriano et al., 1996). A multiple sequence alignment using the GrowTree phylogram showed that TCDVd is related to PSTVd, but formed a separate branch (Fig. 4). Tomato has been reported to be the natural host of two viroids, tomato apical stunt (TASVd) and tomato planta macho (TPMVd) (Kiefer et al., 1983). TCDVd has 73% and 83% sequence identity to these viroids, respectively and was not grouped with them (Fig. 4).

When compared to PSTVd, TCDVd has nucleotide changes in all five domains (Keese & Symons, 1985) of the viroid molecule. However, the Variable (V) and Terminal Right (TR) domains of TCDVd have 59% and 89% sequence identities, respectively, while the remaining molecule has over 97% identity to PSTVd. Since the V domain of PSTVd is known to contain both sequences that are important for replication and accumulation of viroid as well as the ‘premelting region 3’, which is involved in the control of breakdown of the native structure in vitro (Riesner, 1991; Hu et al., 1996), TCDVd, with
its drastically different V domain, provides an alternative structure for experimentation.

When the $T_R$ sequences of TCDVd are compared with members of the genus Pospiviroid, various PSTVd isolates exhibit less than 90% identities, and TASVd, MPVd and Columnnea latent viroid exhibit 93% identities. This indicates that the $T_R$ in TCDVd could have been derived from viroids other than PSTVd.

In addition to the extensive changes in the V and $T_R$ domains when compared to PSTVd, TCDVd has a nucleotide transition of $A_{258} \rightarrow U_{258}$ in the Terminal Left ($T_L$) domain, which is also found in PSTVd-N (Puchta et al., 1990). The addition of $U_{63}$ in the upper strand of the Pathogenicity (P) domain, the substitution of $G_{311}$ in the lower strand of P domain and the transition $U_{258} \rightarrow A_{258}$ in the lower strand of the CCR are other changes in TCDVd. Nucleotide 258 is part of an ultraviolet light-sensitive structural element that is present in RNA molecules of hepatitis delta virus. The hood of circular RNA pathogens: viroids, circular satellites and the hepatitis delta virus.

We would like to thank Vanderveen’s Greenhouses, Carman, Manitoba, for providing the diseased samples and discussion of the occurrence of the disease; Mr Dennis Lidgett, Manitoba, for providing the slides of disease symptoms; and Ms Helena Weilguny, Slovenia, for carrying out some of the initial experiments at the Potato Research Centre, Fredericton.

References


Puchta, H., Herold, T., Verhoeven, K., Roenhorst, A., Ramm, K., Schmidt-Puchta, W. & Sänger, H. L. (1990). A new strain of potato spindle tuber viroid (PSTVd-N) exhibits major sequence differences as compared to all other PSTVd strains sequenced so far. Plant Molecular Biology 15, 509–511.


