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Tomato chlorotic dwarf viroid in the ornamental plant *Vinca minor* and its transmission through tomato seed

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Abstract Two novel aspects of *Tomato chlorotic dwarf viroid* (TCDVd) are reported, namely that TCDVd was detected in symptomless plants of *Vinca minor*, a trailing ground cover surviving at subzero temperatures (−12°C); and that TCDVd was seed-borne in tomato and detected in high percentages in tomato seeds and seedlings. Soaking seeds in a low concentration of sodium hypochlorite did not eliminate the viroid. The sequence analysis showed that the TCDVd isolate consists of 360 nucleotides and has sequence identity between 96% to 99% with isolates of TCDVd from other hosts.

Keywords Detection · Hot-climate pathogen · Ornamental plants · Survival in cool climates · Viroids

Viroids are low-molecular weight circular RNA (Diener 1971; Singh and Clark 1971; Sänger et al. 1976) that infect plants causing devastating plant diseases. Currently, there are >30 viroid species known and they are classified into two families, *Pospiviroidae* and *Avsunviroidae* (Flores et al. 2005). Pospiviroids are considered to be hot-climate pathogens (Singh 1983) because they multiply at 25°C to 35°C (Sänger and Ramm 1975) and their generation is inhibited at lower temperatures (Lizàrraga et al. 1980; Paduch-Cichal and Kryczynski 1987).

Vinca minor, commonly known as myrtle, creeping myrtle, periwinkle or vinca, is often grown as an ornamental ground cover in the southern USA and western Canada. A viroid was isolated from *V. minor* plants that had survived winter temperatures (November to March, 3 to −12°C). Presently, no *Pospiviroid* surviving at such low temperatures in perennial ornamental plants has been reported. Therefore, it was of interest to determine viroid multiplication and molecular composition of a pospiviroid from *V. minor* which survives at cooler temperatures.

The genus *Pospiviroid*, in the family *Pospiviroidae*, contains nine viroid species and several of them, including *Tomato chlorotic dwarf viroid* (TCDVd; Singh et al. 1999), infect ornamental plants (Singh and Teixeria da Silva 2006). In recent years, TCDVd has been encountered in more hosts and from different countries than any other *Pospiviroid* species (Verhoeven et al. 2004; Singh et al. 2006b; James et al. 2007; Verhoeven et al. 2007; Matsushita et al. 2008), ranging from ornamental plants to some commercial greenhouse tomatoes (unpublished observations). The occurrences of TCDVd in commercial greenhouses have raised the possibility of seed transmission of viroid in some host plants. *Potato spindle tuber viroid* (PSTVd), a closely related viroid to TCDVd (Singh et al. 1999) is known to be seed-transmissible in potato and tomato (Benson and Singh

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1964; Singh 1970), in *Scopolia sinensis* (Singh and Finnie 1973), and in true potato seed (TPS) (Cammack and Harris 1973; Fernow et al. 1970; Grasmick and Slack 1986; Singh et al. 1988, 1991). The percent infection of PSTVd in TPS could range from 0% to 100% (Fernow et al. 1970) and in individual fruit from 35% to 67% of seeds (Singh et al. 1992).

Perennial ornamental plants obtained from a local nursery as rooted cuttings imported from the USA were planted outdoors in 2005. Plants which survived winter conditions were sampled for the presence of viroids in 2006 and 2007. *Vinca minor* survived the winter without any noticeable damage to plant parts. The leaves remained dark green during the winter months and new buds started appearing within weeks of snow melt (April to May). New shoots from each plant were used for RNA extraction (Singh et al. 2006a). The method was modified to improve the nucleic acid yield. Briefly, plant sap (150–200 μl) was collected by passing the leaves and vine samples through a tissue grinder (Electrowerk, Behncke and Co. Hanover, Germany) and collecting sap in a microcentrifuge tube containing 300 μl of extracting solution (50 mM NaOH, 2.5 mM EDTA). The resulting extract was centrifuged at 12,000×*g* at 4°C for 15 min. From the supernatant, instead of spotting on nitrocellulose membrane as described (Singh et al. 2006a), RNA was precipitated with 1 vol of isopropanol in the presence of 0.1 vol of 3 M sodium acetate (−20°C overnight). The precipitate was collected by centrifugation (12,000×*g* at 4°C), washed with 70% ethanol, vacuum-dried, and dissolved in 1,000 μl of sterile distilled water.

Reverse transcription was performed as described (Singh et al. 2006a) using 2.5 μl RNA preparation and the *Pospiviroid* reverse primer. For PCR amplification, 2 μl cDNA was added in 23 μl solution mix as described (Singh et al. 2006a) with 0.1 μg of the forward and reverse primers for *Pospiviroid* (5′-ATTAATCCCCGGGGAAACCTGGAG-3′ and 5′-AGC TTC AGT TGT TTC CAC CGG GT-3; Bostan et al. 2004) and for full-length TCDVd (5′-AAA CAG GAG TAA TCC CGT GTA G-3′ and 5′-CGC CTT CCA CAA GCT CCC TGC-3′). For *Potato* PSTVd full length amplification, the primers were (5′-ATCCCCGGGGAAACCTGGAGCGAAC-3′ and 5′-CCC TGA AGC GCT CCT CCG AG-3′). Amplified products were analysed by electrophoresis in a 2% agarose gel containing 0.5 μg ml^{-1} ethidium bromide and photographed under UV illumination with an imaging system (FluorChem, Alpha Innotech Inc., San Leandra, CA, USA).

Amplified products were cloned using a TOPO TA cloning kit for sequencing (Invitrogen Life Technologies, Carlsbad, CA, USA). Plasmids were purified with QIAprep Spin miniprep kit (Qiagen, Mississauga, ON, Canada). The nucleotide sequence of the three recombinant plasmids was obtained by automatic sequencing (NRC Plant Biotechnology Unit, Saskatoon, SK, Canada). Multiple alignments of nucleotides were obtained using NCBI's BLAST programme. The nucleotide sequences were deposited in GenBank database under the accession number EU625577.

The initial indication that *V. minor* contained a viroid was discerned from RT-PCR of leaf samples using the *Pospiviroid* primer pair, which is known to detect several pospiviroids (Bostan et al. 2004; Singh et al. 2006a, b). Occasional testing of leaves of infected *V. minor* plants in February (−12°C) and routine sampling from July to November showed the presence of a high concentration of viroid in all above ground plant parts.

Specific identification of the viroid was made with full-length primer pairs of each viroid species and nucleotide sequence analysis. The TCDVd isolate from *V. minor* was 360 nucleotides in size, similar to the two tomato isolates from Canada (Singh et al. 1999, acc. no. AF162131) and from The Netherlands (Verhoeven et al. 2004, acc. no. AY372399). However, while the *V. minor* isolate is 99% identical to the Canadian tomato isolate, it is only 96% identical to the Dutch tomato isolate. The latter tomato isolate has most of the nucleotide differences in the lower strand of the T1 (left terminal) domain (Keese and Symons 1985) and may indicate a different origin for the Dutch isolate than the Canadian TCDVd. Another TCDVd isolate in tomato from Japan (Matsushita et al. 2008, acc. no. AB329668) consisting of 359 nucleotides was 98% identical to *V. minor* isolate.

Three viroid isolates from Petunia, each of 359 nucleotides in size, and another isolate from *Brugmansia* (Verhoeven et al. 2007, acc. no. 626530), 356 nucleotides, had sequence identities of 96–97% and 96%, respectively, suggesting the isolate from *V. minor* belongs to the TCDVd species. However, there is no apparent sequence 'marker' to indicate the stability of *V. minor* isolate of TCDVd at cooler

temperatures. To determine if the viroid integrity under cold temperatures was particular to the viroid species rather than the ability of the host to survive at cooler temperatures, *V. major* plants infected with *Chrysanthemum stunt viroid* (CSVd) and grown outdoors were also monitored during the same period. *Vinca major* differs from *V. minor* in terms of winter persistence in that *V. major* loses all above ground material during winter, and grows back from carbohydrate root reserves in the spring. CSVd was detected in high concentrations from all new growth shoots sampled between July and November. The nucleotide sequence identity of CSVd grown at warmer temperatures (25–30°C) to that grown under colder conditions was 99%, suggesting that the observed variation is not associated with adaptation to a different temperature regime.

The TCDVd isolate from *V. minor* was transmitted by manual inoculation to tomato (cv. Sheyenne) and *Nicotiana glutinosa* seedlings, and both developed symptoms as previously observed (Singh et al. 1999). In inoculated tomato plants, stereotypical symptom expression of viroid infection such as mild bunching of apical leaves, shortening of internodes, and chlorosis of leaves were evident within 6 weeks post-inoculation. Similarly, RT-PCR testing also confirmed the presence of the viroid in such plants. Tomato plants were monitored for six additional weeks for plant symptom intensification and observation of fruit symptoms. In TCDVd-infected plants, further chlorosis and stunting of top growth was observed and fruit size became progressively smaller. Transfer of the viroid from tomato to tomato resulted in increased severity of symptoms and early appearance (18–21 days) of symptoms. The nucleotide sequence from the infected tomato RNA was 99% identical to the original *V. minor* sequence; thus, the increased concentration of the viroid is the most likely cause of the early and severe symptoms.

Since fruits from infected tomato plants were noticeably affected by the viroid and were progressively reduced in size, tests were made to determine if viroid is present in the seeds of *V. minor*, *N. glutinosa* and tomato. In spite of numerous contacts with suppliers of nursery plants in North America, no seed of *V. minor* could be obtained. Therefore, attempts were made to test the seed originating from tomato cv. Sheyenne and *N. glutinosa* plants infected for 2–3 months with the *V. minor* isolate of the TCDVd. Seeds from both plant types were collected after fruits were ripened (tomato) or dried (*N. glutinosa*). Water-diluted tomato pulp containing seeds obtained from three to six fruits were incubated for 3 days at room temperature (mild fermentation), washed with water until seeds were free of pulp and then dried at room temperature for 3–6 days. From this bulked seed, individual seeds were soaked in 30 μl of extracting buffer, incubated overnight and then ground using a pellet pestle. For seedling assays, another sample was germinated from the bulked seed source and seedlings were grown in the greenhouse. Leaves were sampled for viroid presence when the plants were at a five to six leaf stage. Both seeds and seedlings were tested for the presence of viroids separately. As a control for seed transmission studies, tomato seeds from PSTVd-infected plants were similarly treated and tested.

In a preliminary test, 50 tomato seed extracts from TCDVd-infected plants were individually tested by RT-PCR using the eluant from spotted nitrocellulose membrane (Singh et al. 2006a). Most of the viroid bands were faint, indicating a lower concentration of the viroid. Therefore, the RNA extraction procedure as reported (Singh et al. 2006a) was modified by incorporating an overnight precipitation of RNA. When 50 seeds obtained from the large tomato fruits infected 11 weeks post-inoculation with *V. minor* isolate of TCDVd were tested, 16 seeds (26.7%) were positive for TCDVd (Table 1). In contrast, about 60% of tomato seeds from PSTVd-infected plants (for longer duration) used as a control were found positive for PSTVd in the same test (Table 1). In the next test, tomato fruits from TCDVd plants infected for 15 weeks were collected on the basis of fruit shapes e.g., ‘round’ or ‘deformed’ and seeds were extracted as before. The RT-PCR test showed that both types of fruits contained seeds which were highly seed-borne (Table 1). The round fruits showed 85.5% and the deformed fruits had 94.4% infected seeds. Since there was not a wide difference in seed-borne nature of seeds from round or deformed small fruits, in the next test they were combined and tested together. Of the 100 seeds tested from small fruits, 86% contained seed-borne TCDVd (Table 1). Interestingly, the small fruits had only four to seven seeds in contrast to 30–40 in the large fruits. In another test, 50 seeds from small fruits infected for 15 weeks were grown and seedlings were tested 6 weeks post-germination, when they were 5–6 cm tall. Viroid presence was detected

Table 1 Presence of *Tomato chlorotic dwarf viroid* in tomato seeds and seedlings from infected plants

Sample type	No. of samples	TCDVd detected	% Infection	Infection (weeks)
Seed (large fruit)	60	16	26.7	11
Seed (round)[a]	36	31	86.1	15
Seed (deformed)[b]	34	36	94.4	15
Seed (small fruits)[c]	100	86	86.0	15
Seedlings (small fruits)	50	40	80.0	15
PSTVd (control)	40	24	60.0	11
PSTVd (control)	6	6	100.0	15

[a] Fruits were round in shape as normally seen in cv. Sheyenne.

[b] Fruits were flat and uneven.

[c] Round and deformed combined.

by RT-PCR in 40 seedlings. The RT-PCR–positive seedlings were grown for an additional 6 weeks, when mild bunchy-top symptoms in the top part of plant or lateral shoots were clearly visible. In another approach, the infectivity of tomato seed extract was tested by inoculating the remaining RNA preparation of 12 TCDVd-positive seeds after the RT-PCR test to healthy tomato seedlings. Six of the 12 seedlings developed mild symptoms. The RT-PCR test showed faint viroid bands for nine of the 12 seedlings. For *N. glutinosa*, ten batches of 100 seeds per batch from the same bulked seed source and 17 batches of seedlings (five in each batch), were processed and tested for the seed-borne nature of viroid. No viroid bands were detected in seeds or seedlings tested.

It has been shown earlier that incubation of PSTVd RNA in 1% sodium hypochlorite solution for 15 sec destroys the RNA structure when assessed by return polyacrylamide gel electrophoresis (R-PAGE) (Singh and Boucher 1987) assay (Singh et al. 1989). Therefore, an attempt was made to determine if prolonged incubation of PSTVd or TCDVd-infected tomato seed in a dilute solution of sodium hypochlorite would be effective in destroying the viroid RNA in the intact seed. Tomato seeds obtained from PSTVd and TCDVd-infected plants were soaked for 7–9 days in a filter paper-lined Petri dish containing water or low concentrations (0.125, 0.25, and 0.50%) of sodium hypochlorite. In the first assessment, only 0.25% concentration was tested. Of the 49 tomato seeds soaked in water, 42 were detected as TCDVd-infected (85.7%), while 33 of the 44 in sodium hypochlorite, were identified as TCDVd (75.0%). In the next test, 12 seeds were used for each treatment (water, 0.125, 0.25 and 0.5%). PSTVd was detected in 25.0% to 83.3% of seeds, irrespective of treatment. Similarly for TCDVd, the percentage of TCDVd in seed extracts ranged from 66.6% to 100% indicating that soaking in sodium hypochorite solution did not eliminate the viroids from seed. The experiment was repeated with ten and 20 seeds per treatment. The results were essentially the same as above.

This study demonstrates that at least two pospiviroids can persist at subzero temperatures provided the plant tissues remain alive. This is the first report of any pospiviroid survival at subfreezing temperatures. In addition, the finding of TCDVd in a widely used groundcover which can flourish in extremes of environmental conditions, may contribute to the spread of this viroid and should be avoided near tomato cultivation. The finding of seed transmission of TCDVd in tomato in the current study is of significance and may explain the recent occurrences of the viroid in several greenhouse-tomato production systems. However, other isolates of TCDVd have not been tested in this study and therefore cannot be generalised. The initial description of TCDVd (Singh et al. 1999) reported failure of seed transmission in seeds of different solanaceous hosts including tomato when tested by R-PAGE. Since RT-PCR is more sensitive than R-PAGE, another attempt was made to determine whether the *V. minor* isolate of TCDVd is seed-transmissible. Even with the RT-PCR test, the TCDVd bands were faint in the majority of cases unless the quality of viroid RNA preparation was improved. Therefore the earlier failure of seed transmission could be due to the low concentration of viroid in seeds. In light of this result, it is tempting to speculate that the seriousness of the current viroid problem in greenhouses may be driven by the

greenhouse production technology. For example, once a viroid is introduced through infected seeds or seedlings in a greenhouse operation, the greenhouse environmental conditions (high temperature and humidity) would favour viroid multiplication, resulting in high concentrations of viroid in new growth. The practice of removing young side-shoots from the main stem of tomato plants in commercial greenhouses and maintaining plants for 9–10 months may also contribute to the spread of the viroid through contamination. Therefore, the prevention of viroid introduction through viroid-tested seed or seedlings may provide the best management strategy for the current problems in greenhouse tomato production.

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