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Molecular and biological characterization of a severe isolate of *Tomato chlorotic dwarf viroid* containing a novel terminal right (T_R) domain sequence

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Abstract *Tomato chlorotic dwarf viroid* (TCDVd) manually inoculated to transgenic (cv.'Desiree') potato plants containing antimicrobial cationic peptides failed to develop symptoms in above ground plant parts, but infected tubers were symptomatic. Plants from the infected tubers (second generation plants) emerged as either severely stunted (bushy stunt isolate, BSI) or tall and symptomless. Molecular characterization of BSI isolates showed TCDVd sequence variants 95 to 98% identical to TCDVd sequences from the database, while a viroid variant identical to TCDVd type isolate (acc # AF162131) was cloned from symptomless plants. The TCDVd BSI variants had novel U165C, GU177-178AA, and UCAC181-184CUUU nucleotide substitutions in the terminal right (T_R) domain of the viroid molecule. The cloned viroid cDNAs of the BSI were infectious to experimental (cv. 'Sheyenne') tomato plants causing stunted plants with profuse auxiliary shoots. Visual evaluation of the susceptibility of the BSI to 18 potato and 21 tomato cultivars revealed severe symptoms in most cultivars of both species. The progeny variants accumulating in each potato and tomato cultivar exhibited the same novel T_R domain in most cultivars, with only a slight variation in a few. The severity of the stunting symptoms induced by TCDVd from BSI isolates in both potato and tomato cultivars has not been noted previously with other TCDVd isolates and, as such, it is proposed that this new isolate be recognized as a distinct genotype. Emergence of this type of sequence variant in commercial fields or commercial tomato greenhouses could potentially cause relevant losses in both crops.

Keywords Evolution of viroid · Extreme stunted plants · Sequence variants · Viroid strain · Variation of symptoms

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Introduction

Tomato chlorotic dwarf viroid (TCDVd) belongs to the genus *Pospiviroid* and the family *Pospiviroidae* (Flores et al. 2005). It was first reported from Canada (Singh et al. 1999) on greenhouse tomato (*Solanum lycopersicum,* formerly *Lycopersicon esculentum*) plants exhibiting chlorotic leaves and dwarfing symptoms. Since then, it has been reported on tomatoes from the Netherlands (Verhoeven et al. 2004), Japan (Matsushita et al. 2008), France (acc. # EU729744) and the USA (Ling et al. 2009). In addition to the tomato crop, TCDVd has also been isolated from several greenhouse grown ornamental plants (e.g., *Verbena* x *hybrida* (Singh et al. 2006a), *Petunia* x *hybrida* (Verhoeven et al. 2007; James et al. 2008), *Brugmansia sanguinea* (acc. # EF 626530) and *Vinca minor* (Singh and Dilworth 2008). TCDVd can infect plants in the families of *Apocynaceae*, *Compositae*, S*olanaceae* and *Verbenaceae* (Matsushita et al. 2009). Thus, TCDVd is well-adapted to survive in greenhouse growing conditions and can spread rapidly as evidenced by an increase of over 3,000 symptomatic tomato plants within a 6-month period (Matsushita et al. 2008).

TCDVd RNA ranges from 356 nucleotides (nt) (acc. # EF626530) to 361 nt (acc. # FJ822878) and like other pospiviroids, forms a circular secondary structure. The 11 full-length sequences of TCDVd available from the database (as of July 2009), can be grouped into two genotypes. Irrespective of the original infected host plant, nucleotide sequences of ten isolates are 97–100% identical to the type isolate (acc. # AF162131), while one isolate from tomato possessing 96% identity to the type isolate has been considered the second genotype (Ling et al. 2009).

In this paper we report that the mechanical inoculation of a transgenic potato line resulted in the emergence and replication of a TCDVd sequence variant which induces profuse auxiliary shoots and extreme stunting in both potato and tomato plants. Molecular and biological characterization of several TCDVd isolates inducing severe stunting, termed bushy stunt isolates (BSIs), showed that these are the most severe isolates for both potatoes and tomatoes under experimental greenhouse conditions. They have not been evaluated under field conditions or in commercial tomato greenhouses.

Material and methods

Transgenic and non-transgenic potato (cv. 'Desiree'), tomato and potato cultivars, viroid culture and inoculation

Transgenic potato containing antimicrobial cationic peptides has been shown to provide antibacterial, antifungal and antiviral resistance to potato diseases (Misra and Bhargava 2008). One transgenic line of cv. 'Desiree' (M3-26) originally referred to as (MsrA3) (Osusky et al. 2004), was obtained as tubers and the resulting plants were evaluated for their reaction to viroid infection. The non-transgenic 'Desiree' and other cultivars obtained as tissue culture plantlets (Plant Propagation Center, NB Department of Agriculture and Aquaculture) were also tested for TCDVd reaction. Viroid cultures were maintained in tomato cultivar 'Sheyenne' by serial transfers to young tomato plants (Singh et al. 2006b). Inoculum from leaves was prepared in an extracting buffer (50 mM NaOH, 2.5 mM EDTA) at a tissue to buffer ratio of 1:5 (W/V). The homogenate was rubbed on 3–5 fully developed leaves. Inoculated plants were maintained in a greenhouse at 25 to 31°C temperatures, relative humidity over 70% and a 14 h day-length with supplemental light. Leaves from inoculated plants were tested by RT-PCR and bioassay on tomato cultivar 'Sheyenne'.

Twenty tomato cultivars available locally, in addition to Sheyenne, and 18 potato cultivars widely grown in North America were evaluated for their reaction to BSI-TCDVd. Eight tomato plants and four potato plantlets from each cultivar were manually inoculated using buffered leaf sap prepared from infected tomato or potato leaves. Visual symptoms were recorded periodically for 8 weeks. Seeds from mature tomato fruits were extracted, dried and tested for seed transmission. Potato tuber symptoms were also recorded immediately after harvesting and during cold storage.

Nucleic acid extraction and RT-PCR

Leaves, tubers and seed tissues were used for RNA extraction (Singh et al. 2006b). Briefly, plant sap (150–200 μl) was collected by passing leaves and tuber pieces through a tissue grinder (Electrowerk, Behncke and Co. Hanover, Germany) and collecting

sap into a micro-centrifuge tube containing 300 µl of extracting solution (50 mM NaOH- 2.5 mM EDTA). The resulting extract was centrifuged at 12,000 x *g* at 4°C for 15 min and from the supernatant, 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 x *g* at 4°C), washed with 70% ethanol, vacuum dried, and dissolved in 1,000 µl of sterile water. Tomato seeds were extracted as described using a mechanical pestle (Singh and Dilworth 2008).

Reverse transcription was performed as described (Singh et al. 2006b) using 2.5 µl nucleic acid extract and 0.1 µg of the PSTVd full-length reverse primer (5′-ATCCCCGGGG AAACCTGGAGCGAAC-3′ Forward and 5′-CCCTGAAGCGCTCCTCCGAG-3′ Reverse) in a 10 µl final volume. PCR amplification was carried out (Singh et al. 2006b) with 2 µl of cDNA added to 23 µl solution mix. Amplified products were analyzed by electrophoresis in a 2% agarose gel containing 0.5 µg/ml ethidium bromide and photographed under UV illumination with an imaging system (FluorChem, Alpha Innotech Inc.).

Nucleotide sequence determination and infectivity of cDNAs

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 was determined using automated sequencing. Three clones were sequenced for each sample. Pair-wise comparison of nucleotides was performed using NCBI's BLAST program. The multiple alignments of nucleotide sequences were carried out with the ClustalW program (Thompson et al. 1994). The sequence obtained from the transgenic potato line containing novel substituted nucleotides in the T_R domain has the accession number GQ169709.

For infectivity tests, three original BSI-potato clones were inoculated to tomato (parental clones) and transferred 5 times consecutively over a six month duration to tomato seedlings (the progeny clones) for a total 18 tomato samples. From these clones, 3 parental and 6 progeny clones selected at random were inoculated for infectivity tests. Viroid from these 9 infected-tomato plants was transferred once more to another 9 tomato seedlings (tomato transfers). All of these samples along with 3 clones of symptomless potato and 3 BSI-tobacco were sequenced (Table 1). For the inoculation of cDNA clones, inocula were rubbed on cotyledons of tomato seedlings using 20 µl REact buffer (Invitrogen) after *Eco*R I treatment. The control plants were mock-inoculated with buffer.

Results

Stunting symptoms in transgenic potato and non-transgenic hosts

The following five inoculation experiments were carried out to determine the transmission, symptomology and viroid concentration. Twelve plants of cv. 'Desiree' (transgenic line M3-26) were inoculated with TCDVd as part of a viroid resistance evaluation study. None of the inoculated transgenic plants exhibited visible symptoms in above ground plant parts during current season of infection, although all inoculated plants were infected as determined by RT-PCR. Tubers from the infected plants were elongated and spindly, a symptom observed with TCDVd (Singh and Dilworth 2008). A total of 86 transgenic potato plants originating from the infected tubers (second generation plants), exhibited a range of stunting symptoms (Fig. 1). Forty plants were moderate to extremely stunted, with smaller leaves and attained a height of only 15 to 22 cm (Fig. 1 a, b) and were termed bushy stunt isolates (BSI), while the remaining (46 plants) were almost symptomless and over 30 cm tall with larger leaves (Fig. 1c).

TCDVd from 21 BSI potato plants and from 19 symptomless ones were transferred to two 'Sheyenne' tomato plants each manually and by graft-inoculation. Symptoms of extreme stunting were observed in the tomato plants inoculated with TCDVd from the BSI isolates, while mild bunchy-top symptoms, typical of previously reported TCDVd isolates (Singh et al. 1999), were observed in tomato plants inoculated with the 'symptomless' potato sources. RT-PCR analysis indicated that all tomato plants (in both groups) were infected by the viroid. However, the PCR-band intensity was weaker in the plants infected with the 'symptomless' sources, suggesting differential

Table 1 Number of sequenced clones and nucleotide substitutions in the T_R domain of sequenced variants from BSI-isolates

Plant Samples	No. of clones	Nucleotide substitutions in T_R domain		
Acc. AF162131	TCDVd	165 U	177-178GU	181-184UCAC
BSI-potato[a]	3	C	AA	CUUU
Symptomless potato[b]	3	U	GU	UCAC
BSI-tomato[c]	5	C	AA	CUUU
BSI-tobacco[d]	3	C	AA	CUU-
Infectious clones[e]	9	C	AA	CUUU
Tomato transfers[f]	9	C	AA	CUUU

[a] Viroid clones from BSI-potato (original isolates)

[b] Viroid clones from symptomless potato

[c] Viroid clones from original BSI-potato transferred to tomato

[d] Viroid clones from original BSI-potato transferred to tobacco

[e] Three viroid clones from BSI-tomato (parental) and 6 clones from consecutively five times transferred to tomato (progeny)

[f] Infectious clones once transferred to tomato (progeny)

biological properties of TCDVd populations from BSI and symptomless potato sources. Another set of 12 potato plants (transgenic line M3-26) was inoculated with the potato leaf extract from BSI-isolates to determine symptomology in the current season and in the second generation plants. There were no symptoms in the above ground plant parts in the current season, but all of the second generation plants developed severe stunting and none were symptomless, suggesting that elicitation of BSI symptomology is generally induced in the second generation of transgenic plants, as initially observed in the first experiment. However, this is not always the case. In fact in a repeat experiment with 12 potato plants (transgenic line M3-26) the second generation plants from small and spindly tubers infected by the viroid were not stunted. In this case, all plants were symptomless type (Fig. 1c), indicating that the transgenic plant may not be the factor in causing stunted plants.

TCDVd from BSI isolates was also transferred to two species of tobacco (*Nicotiana* spp) to discern its response in these hosts. BSI inoculation caused flower colour break in *N. glutinosa*, as observed with typical TCDVd (Singh 2006), whereas infected tobacco (*N. tabacum*) plants remained symptomless, as has been currently observed with TCDVd isolated in Japan (Matsushita et al. 2009). The viroid was readily detected in both hosts (*N. glutinosa* and *N. tabacum*) by RT-PCR and by biological assays using tomato

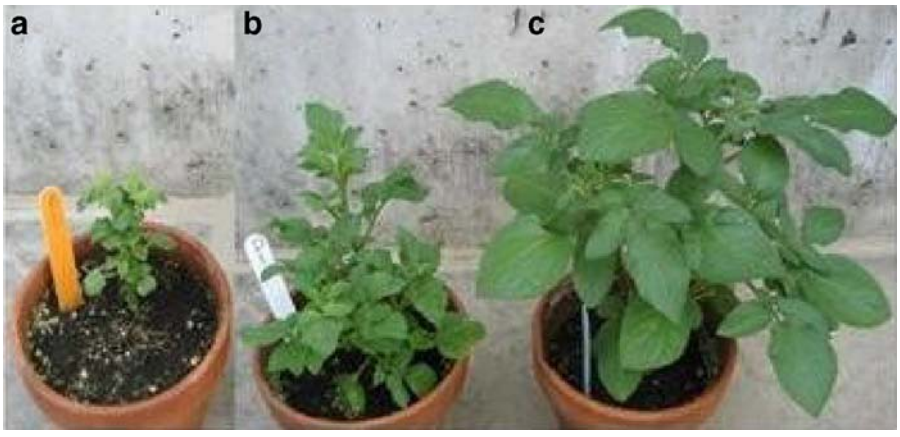

Fig. 1 Stunted and symptomless potato cv. Desiree plants infected with Bushy stunt isolate of Tomato chlorotic dwarf viroid. **a** Extremely stunted; **b** moderately stunted and **c** symptomless plants

plants as indicator hosts that, after the inoculations, developed extreme stunting symptoms.

Nucleotide sequence and BSI-TCDVd identification

Nucleotide sequences of 3 clones each of BSI from the original transgenic 'Desiree' potato plants (BSI and 'symptomless'-type plants), five BSI consecutive transfers to 'Sheyenne' tomato and three to tobacco plants, showed that the viroid consisted of 360 nt, having 98% identity to TCDVd (acc. AF162131). Multiple alignments of the sequences from these hosts showed a consistent pattern of nt substitutions of seven nucleotides in the T_R domain of the BSI molecule (Fig. 2B) compared to TCDVd acc. AF162131 (Fig. 2A). The nt substitutions for the BSI molecule consisted of U165C, GU177-178AA and UCAC181-184CUUU in the terminal right domain loop portion of the TCDVd molecule (Fig. 2B). There were no substitutions in the viroid RNA from symptomless plants (Table 1), which was identical to acc. AF162131 (Fig. 2A). The substitutions do not change the secondary structures; however, the paired bases were altered (Fig. 2B).

Nine cDNA full-length clones originally from infected 'Desiree' potato and subsequently from 'Sheyenne' tomato were inoculated to additional tomato plants to determine their infectivity and sequence stability (Table 1). The BSI clones irrespective of the host species used, were infectious and all of them possessed the altered nucleotides of the T_R domain loop (Table 1). Transfers of the BSI cDNA clones, originally obtained from potato (parental clones) to new tomato seedlings (the progeny clones) exhibited severe stunting symptoms. The nucleotide sequences were identical to the parental sequences, indicating sequence stability (Table 1).

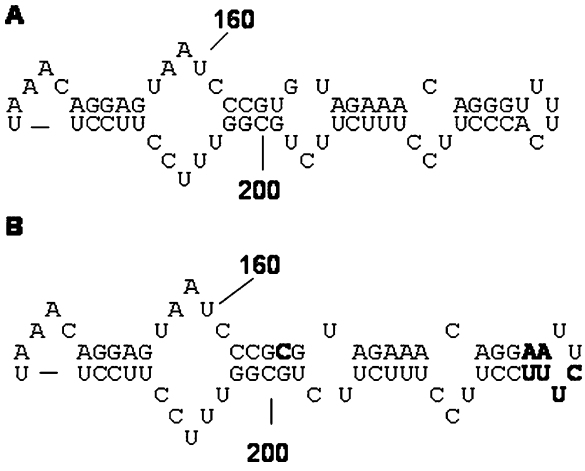

Fig. 2 The nucleotide sequence of the Terminal right (T_R) domain of Tomato chlorotic dwarf viroid isolates. **A** Acc. AF162131; **B** From the Bushy stunt isolate of TCDVd. The substituted nucleotides are marked in bold in B

Susceptibility of the potato and tomato cultivars to 'bushy stunt' isolate

The extreme stunting symptom of the BSI-TCDVd was observed in two economically important crop plants; namely potato (cv. 'Desiree' transgenic line) and the experimental tomato (cv. 'Sheyenne'). Since TCDVd has been observed to cause severe symptoms in commercial potato and tomato cultivars (Singh et al. 1999; Verhoeven et al. 2004; Matsushita et al. 2008, 2009), it was necessary to evaluate the effect of the BSI-TCDVd on commercial potato and tomato cultivars used in Canada.

Eighteen potato cultivars (Table 2) were evaluated for the effect of BSI-TCDVd. In contrast to transgenic Desiree, where symptoms in above ground plant-parts were not observed in current year of infection, all non-transgenic cultivars, including Desiree, developed smaller leaves and shortened internodes at 4 weeks post inoculation (wpi). At 6 wpi, all inoculated plants had upright growth typical of TCDVd (Singh et al. 1999) and were infected as determined by RT-PCR. The plants were bushy and all nodes had started developing shoots with extremely shortened internodes and leaves (Fig. 3a). At 8 wpi, most varieties were showing stunting, ranging from 5 to 56% compared to the healthy controls, except cv. 'LaRouge' (Table 2). All cultivars exhibited elongated and spindly tubers with growth cracks (Fig. 3b) and had severely reduced yields, except LaRouge (Table 2). After cold storage of tubers, BSI-TCDVd infected tubers of most cultivars developed densely crowded or branched sprouts (Fig. 3c), which developed into multi-stem bushy plants. TCDVd progeny variants accumulating in the inoculated hosts were sequenced. The nucleotide substitutions typical of TCDVd from BSI isolates (Fig. 2B) were present in TCDVd variants from all potato cultivars with minor variations in some of them. In variants from 3 cultivars (Goldrush, Ranger Russet and Shepody)

Table 2 Symptoms and yield reduction in potato cultivars inoculated with a bushy stunt isolate of TCDVd and nucleotide substitutions found in T_R domain of the respective progeny variants

Cultivars	Stunting	Upright	Lateral	Yield	nt.165..177-180..181-84[a]		
	(%)	Growth	Shoots	Reduction (%)	U	GUUU	UCAC
AC Chaleur	27	++	++	68	C	AAA .	CUUU
Atlantic	49	++	++	89	C	AA . .	CUUU
Calwhite	29	+	++	65	C	AA . .	CUUU
Cherokee	7	+	++	24	C	AA . .	CUUU
Desiree	24	+	++	20	C	AA . .	CUUU
Goldrush	27	++	++	75	U	AA . .	CUUU
Jemseg	17	++	++	77	C	AA . .	CUUU
Katahdin	5	++	++	14	C	AA . .	CUUU
Kennebec	31	++	++	59	C	AA . .	CUUU
LaRouge	0	–	+	0	C	AAA .	CUUU
Red LaSoda	11	+	++	49	C	AAA .	CUUU
R. Burbank	56	++	++	72	C	AA . .	CUUU
R. Norkotah	7	+	++	80	C	AA . .	CUUU
Ran. Russet	53	++	++	66	U	AAA .	CUUU
Shepody	14	+	++	62	U	AAA .	CUUU
Spunta	23	++	++	49	C	AA . .	CUUU
Superior	27	++	++	51	C	AA . .	CUUU
Yukon Gold	47	++	++	72	C	AA . .	CUUU

[a] nt followed by numbers = nucleotide in TCDVd acc.AF162131

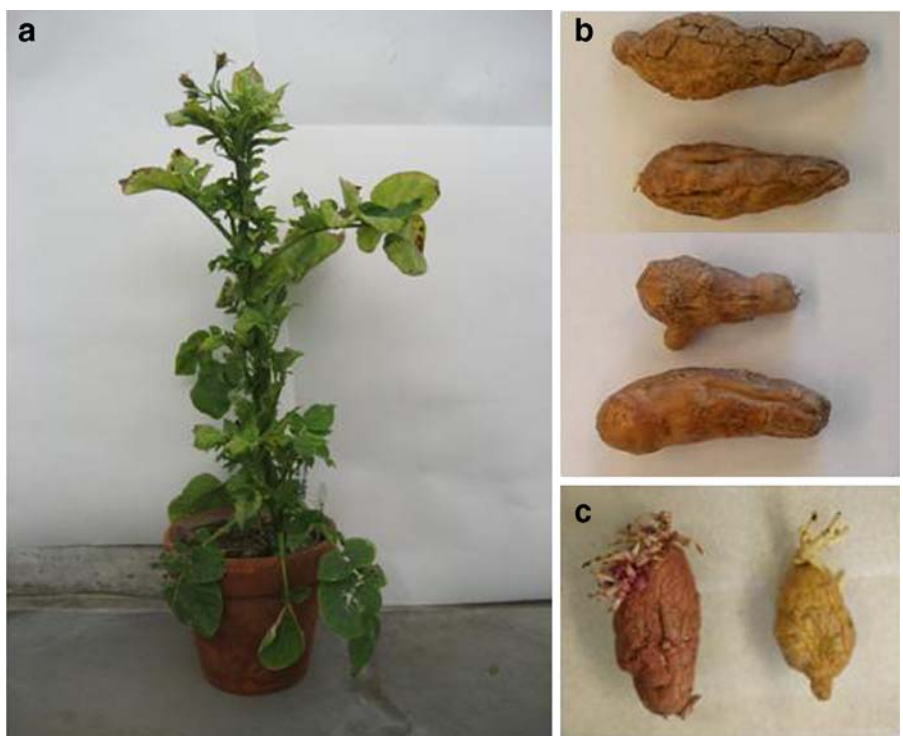

Fig. 3 Symptoms of bushy stunt isolate of Tomato chlorotic dwarf viroid in potato cv. Superior. **a** Late symptoms; **b** spindly tubers; **c** Profuse sprouting after storage

Table 3 Symptoms and yield reduction in tomato cultivars inoculated with a bushy stunt isolate of TCDVd and nucleotide substitutions found in T_R domain of the respective progeny variants

Symptoms of bushy stunt isolate of TCDVd in commercial tomato cultivars							
Cultivars	ST(%)	P&SN	BT	VN	nt.165..177-180..181-184[a]		
					U	GUUU	UCAC
Balcony Charm	27	+	++	+	C	AA ..	.. UU
Big Beef Hybrid	23	++	++	++	C	AA ..	.. UU
Brandywine Red	34	++	++	++	U	AA ..	.. UU
Bush Beefsteak	40	++	++	++	U	AA ..	-. UU
Cherry Roma	30	++	–	++	U	AA ..	CUUU
Christmas Grape	17	–	+	–	U	AA ..	CUUU
Cupid	52	++	–	++	C	AA ..	CUUU
Early Cascade Hybrid	18	++	++	++	U	AA ..	CUUU
Early Girl Hybrid	15	+	++	+	U	AA ..	CUUU
Pink Ponderosa	13	+	+	++	C	AA ..	CUUU
Purple Prince	32	++	++	++	U	AA ..	CUUU
Rainbow Blend	23	+	+	+	C	AA ..	CUUU
Roma V.F.	45	++	++	++	U	AA ..	CUUU
Scotia	35	++	++	++	U	AA ..	CUUU
Starfire Improved	8	++	++	++	U	AA ..	- . UU
Sub Arctic Plenty	24	++	+	++	U	AA ..	- . UU
Sweet 100 Hybrid	0	++	–	+	C	AA ..	- . UU
Sweet Cherry Hybrid	28	++	–	++	C	AA ..	. . UU
Tiny Tim	17	++	+	++	C	AA ..	CUUU
Vita Gold	33	++	++	++	C	AA ..	CUUU
Sheyenne Indicator	61	++	++	++	C	AA ..	CUUU

ST stunting; *P&SN* petiole and stem necrosis; *BT* bunchy-top; *VN* veinal necrosis

[a] nt followed by numbers = nucleotide in TCDVd acc.AF162131

the nucleotide at position 165 was U, while this nucleotide was substituted by C in the remaining ones (Table 2). The 6 nt substitutions in the terminal loop portion of the T_R (Fig. 2B) also varied depending on the cultivar. Variants from thirteen had 6-nt (AA.. CUUU) substitutions in this region in comparison to the type isolate acc. #AF162131, whereas the remaining five cultivars had 7-nt (AAA..CUUU) substitution identified in BSI-isolates described above (Table 2).

Twenty one cultivars including 'Sheyenne' and field-grown, climbing and cherry-type tomatoes were evaluated for the BSI reaction (Table 3). Symptoms such as ruffled leaves and shortening of internodes were apparent within 2 wpi. By the fourth week, symptoms ranged from smaller leaves, veinal, petiole and stem necrosis, to bunching of the top part of the plant and the beginning of auxiliary growth from nodes (Fig. 4a). With increased time, reddening and bronzing of leaves, stunting and profuse auxiliary growth from each node gave a bushy appearance to the plants (Fig. 4b). By this time most of the larger, older leaves became necrotic and dropped off. By the 8th wpi, irrespective of the type of tomato, all cultivars with the exception of cv. 'Sweet 100 Hybrid' were stunted between 8 to 61% compared to their healthy controls (Table 3). The number of fruits on BSI-infected tomato plants was not reduced, but the size of fruits (Fig. 4c) and seeds (Fig. 4d) compared to the TCDVd-infected plants was greatly reduced. Of the 220 seeds obtained from fruits of cvs 'Balcony Charm', 'Early Cascade Hybrid', 'Pink Ponderosa', 'Purple Prince', 'Roma V.F.', 'Rainbow Blend', 'Scotia', 'Sheyenne', 'Starfire Improved', 'Sub Artic Plenty', and 'Sweet 100 Hybrid', 27 seeds tested positive to TCDVd by RT-PCR, confirming seed – borne characteristics of TCDVd (Singh and Dilworth 2008). The nt sequences of TCDVd variants from the individual tomato cultivars showed that most substitutions found to be present in TCDVd from parental BSI-isolates were maintained in TCDVd variants

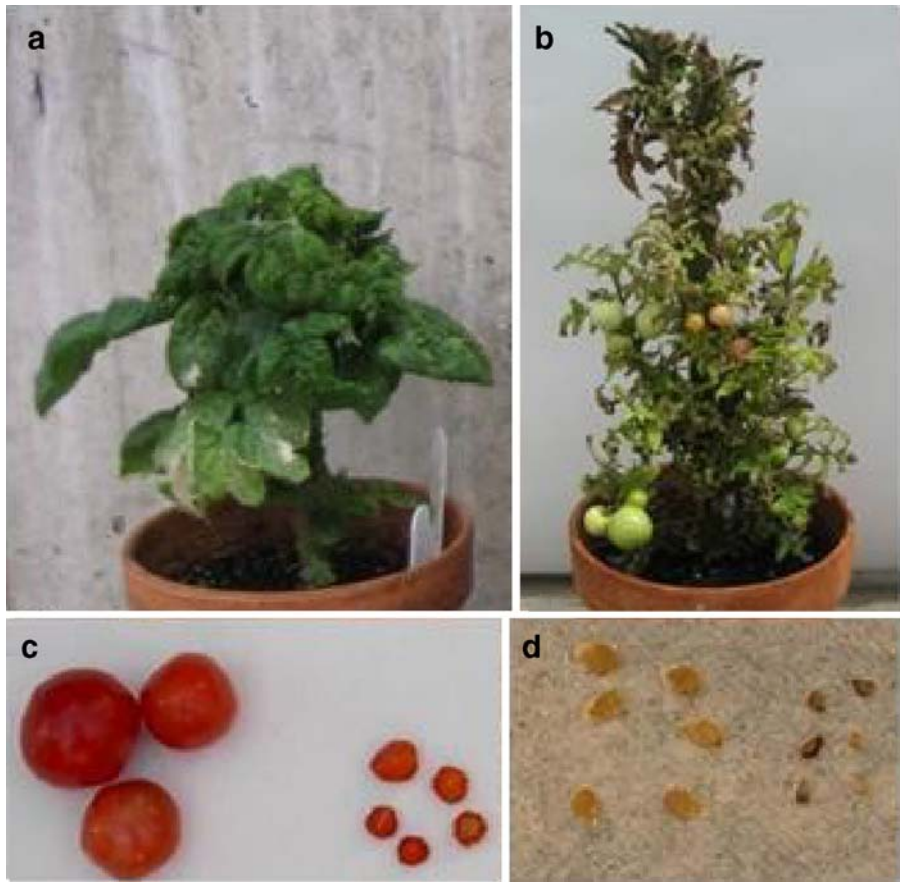

Fig. 4 Symptoms of bushy stunt isolate of Tomato chlorotic dwarf viroid in Sheyenne tomato. **a** Early symptoms; **b** late stage symptoms; **c** Fruits on the left from TCDVd and on the right from the Bushy stunt isolate of TCDVd. **d** Seeds on the left from TCDVd and on the right from the Bushy stunt isolate of TCDVd

from all tested cultivars. However, similar to the potato cultivars, the nt U165C substitution was detected in 10 cultivars, while 13 cultivars had 6 nt (AA..CUUU) substitutions, and the remaining eight cultivars had only 4 nt (AA..-.UU) substitutions, with single nt deletion in some cultivars (Table 3). There was no discernible correlation between any tomato cultivar and a specific nt variation.

Discussion

TCDVd as originally described (Singh et al. 1999) differs significantly from *Potato spindle tuber viroid* (PSTVd) in the Variable and Terminal right domains, which could in large part accounts for the differences in symptom expression between the two viroids; namely, TCDVd is far more aggressive than PSTVd.

The terminal domains of viroids are thought to be extensively exposed to sequence exchanges (Keese and Symons 1985) as exemplified by *Columnea latent viroid* (Singh et al. 1992), *Tomato apical stunt viroid* (Keifer et al. 1983), and *Tomato planta macho viroid* (Keifer et al. 1983), which could derive from recombination events between different viroids including PSTVd and *Citrus exocortis viroid* (Owens et al. 1995). The novel nucleotide substitution pattern detected in the variants from BSI isolates is absent in the T_R region of all the eleven TCDVd sequence variants available from the database, which have been reported from different host plants and from different countries e.g., accessions AF162131 and EU 625577 (Canada), DQ859013, AY372399 and EF626530 (the Netherlands), FJ822877 and FJ822878 (USA), AB329668 (Japan), EF582392 and EF582393 (United Kingdom) and EU 729744 (France). The nearest changes to the above segment in the T_R were found in two TCDVd accessions (acc. # EF626530, U165 is deleted, GU177-178AU and C184U and in acc. # FJ822877, U165C). In addition, no complete identity to this substitution was found in the T_R region of any other viroid species from the database.

Interestingly, the 7 nt substitutions were conserved in the progeny variants accumulating in five consecutive mechanically and graft inoculated tomato cv. 'Sheyenne' and two transfers to transgenic potatoes 'Desiree'. Out of 38 potato and tomato cultivars, the substitution pattern was identical in 13 potato and 13 tomato cultivars, while it varied in the rest of the cultivars (Tables 2 and 3, respectively), probably due to the different genetic make up of the cultivars.

The origin of BSI-TCDVd is not evident. The transgenic potato plants were infected by manual sap-inoculation and the transfer of viroid by sap-inoculation to 'Sheyenne' tomato resulted in one phenotypic, extremely severe, stunting viroid isolate (Figs. 1, 4; Table 1). However, repeat inoculation of the same transgenic potato line failed to reproduce a similar phenotypic TCDVd variant, indicating that transgenic lines may not be a factor in the evolution of the viroid variant. However, the genetic manipulation does affect the expression of the viroid in the current growing season. When comparing transgenic cv. 'Desiree' to the non-transgenic cultivar, expression is latent in the growing season. The nature of viroid interaction with transgenic or genetically different cultivars could also depend on the fact that viroid RNA exists as quasi-species (Gora-Sochacka et al. 1997; Owens et al. 2003), a concept which postulates the existence of a complex, self replicating population of diverse and related entities acting as a whole (Eigen 1993). A similar situation could be postulated in our study, because the TCDVd culture has been maintained in tomato and non-transgenic potato plants for a decade. Therefore, the emergence of a severe isolate of TCDVd could happen by a successful adaptation and dominance of a particular sequence variant already present in the inoculum.

The severity of stunting symptoms of BSI-TCDVd under greenhouse conditions is exceptional. The recent outbreaks of TCDVd in commercial tomato greenhouses have become a serious concern and are resulting in heavy expenditures in eradicating the infection sources. Coupled with the uniformity of stunting symptoms observed in most potato and tomato cultivars with the BSI-TCDVd, the situation becomes unusual. For example, in studies dealing with the host-range of PSTVd, when large numbers of tomato cultivars, hybrids and species (Singh 1966, 1973; Singh and O'Brien 1970) or potato cultivars and species (Bagnall 1972; Singh and Slack 1984; Singh and Crowley 1985) were used for symptom expression, most of them were symptomless with only a few symptomatic for the viroid. With the BSI-TCDVd inoculation, not only did most commercial cultivars of potato and tomato became infected, but they were stunted and produced profuse auxiliary growth with smaller tubers and fruits (Figs. 3, 4). Therefore, attempts should be made to prevent the introduction of BSI type of TCDVd in the commercial tomato greenhouses by implementing a system of tomato seed testing for TCDVd and, if TCDVd infection has taken place, avoid its build-up by rapid eradication.

The seven nt substitutions in the T_R domain represent a major alteration in the BSI-TCDVd molecule and has not been found in any viroid from the public database. Whether this domain is actually involved in the severity of symptoms in potato and tomato has not been determined in this study. Indeed, experiments based on site-directed mutagenesis are needed to conclusively prove the nucleotides involved in the pathogenicity modulation. Along with the novel substitution pattern and the severe symptomlogy induced in potato and tomato by the BSI-TCDVd, it should be considered a distinct genotype of TCDVd.

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