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Incidence and genetic stability of *Potato spindle tuber pospiviroid* in potato in Turkey

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Abstract: The prevalence of *Potato spindle tuber pospiviroid* (PSTVd) infection in randomly selected potato tubers was determined via nonisotopic dot blot hybridization and reverse transcriptase polymerase chain reaction (RT-PCR). In January and February of 2010, 168 seedling tubers of *Solanum tuberosum*, representing 27 cultivars, were received from different seedling potato producers in Turkey. Digoxigenin-labeled RNA probes of PSTVd were synthesized from the cloned PCR products and applied in dot blot hybridization to detect viroids in RNA extracts isolated from dormant potato tubers. PSTVd was found in 7 samples and 6 cultivars (Innovator, Lady Jo, Russet Burbank, Agria, Provento and Konsül). Some RNA samples, although positive by RT-PCR test, did not give a hybridization signal by dot blot assay. The complete genome of a selected isolate (PSTVd-TR) was cloned and sequenced. Primary and secondary structure analysis showed that the RNA genome of the PSTVd-TR isolate (GenBank Accession No. HQ456944) differed from other compared isolates by only a few nucleotides. The highest identity (99%) was in a PSTVd isolate that was identified in *Solanum* spp. in Italy (Accession No. EF459700). The infectivity of PSTVd-TR isolate was shown 2 weeks after the mechanical inoculation of sap extracts onto seedlings of tomato cultivar Joker. The PSTVd infections were proven by RT-PCR assay in symptomless tomato seedlings.

Key words: Dot blot hybridization, molecular characterization, potato, PSTVd

Türkiye'de tohumluk patates yumrularında patates iğ yumru viroidi (PSTVd)'nin saptanması ve genetik stabilitesi

Özet: Ülkemizdeki bazı tohumluk patates çeşitlerinden tesadüfî olarak seçilen dormant patates yumrularında PSTVd enfeksiyonu, radyoaktif olmayan dot blot hibridizasyon ve RT-PCR testleri ile belirlenmiştir. 2010 yılı Ocak ve Şubat aylarında Türkiye'de tohumluk patates üretimi yapan farklı firmalardan, 27 patates çeşidine ait toplam 168 patates yumru örneği temin edilmiştir. PSTVd genomuna spesifik digoksigenin ile etiketli RNA probu, pSPT18 vektörüne aktarılan PCR ürününden sentezlenmiş ve dormant patates yumrularından izole edilen RNA ekstraktlarında etmen viroidi tespit etmede kullanılmıştır. Yürütülen moleküler testlerde toplam 7 numunede (6 çeşit: Innovator, Lady Jo, Russet Burbank, Agria, Provento ve Konsül) PSTVd infeksiyonuna rastlanmıştır. Bazı RNA örnekleri, RT-PCR testinde pozitif sonuç vermesine rağmen, dot blot testinde hibridizasyon sinyali vermemiştir. Seçilen bir izolata ait (PSTVd-TR) genomun tamamı klonlanarak baz dizisi tespit edilmiştir (Gen Bankası Ulaşım No. HQ456944). İzolatın primer ve sekonder nükleotid yapısına uygulanan filogenetik analiz, PSTVd-TR genomunun karşılaştırıldığı diğer izolatlardan sadece birkaç nükleotid düzeyinde farklılık gösterdiğini ortaya koymuştur. PSTVd-TR izolatının, yakın zamanda

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İtalya'da *Solanum* spp.'de tespit edilen PSTVd izolatı (Gen Bankası Ulaşım No. EF459700) ile yüksek düzeyde (% 99) benzerlik gösterdiği tespit edilmiştir. PSTVd-TR izolatının patojenitesi Joker çeşidi domates fidelerine gerçekleştirilen mekanik inokulasyon ile belirlenmiştir. Oluşan infeksiyonun varlığı belirti göstermeyen domates fidelerine uygulanan RT-PCR testi ile doğrulanmıştır.

Anahtar sözcükler: Dot blot hibridizasyon, moleküler karakterizasyon, patates, PSTVd

Introduction

Low-molecular-mass plant pathogens, or viroids, are circular RNA molecules with 246-399 nucleotides (nts) (Singh 1998). Over 40 plant diseases have now been ascribed to viroids in agricultural, horticultural, and ornamental plants (Diener 1987; Singh and Dhar 1998; Hadidi et al. 2003). The nt sequences of almost all viroids, along with their many sequence variants, are known. In some cases, these sequences have been correlated with symptom severity and have been assigned to viroid strains (Visvader and Symons 1985; Schnölzer et al. 1985; Herold et al. 1992; Gora et al. 1994; Singh et al. 1999).

Potato spindle tuber pospiviroid (PSTVd) is infectious, unencapsidated, small, circular, single-stranded RNA with considerable secondary structure, and it is capable of autonomous replication when inoculated into a host. In potato, it is commonly 359 nts in length (Gross et al. 1978). PSTVd, with its numerous well-characterized strains and isolates, was the first viroid to be discovered (Diener 1971; Singh and Clark 1971). It was structurally characterized (Sanger et al. 1976) and sequenced (Gross et al. 1978). It is the type species of the genus *Pospiviroid* (Flores et al. 1998; Singh et al. 1999).

Symptoms of PSTVd depend on strain, cultivar, and environment and may vary from severe symptoms (reduction in plant size, uprightness, and dark green and rugose leaves) to mild and symptomless infections. Tubers may be reduced in size, misshapen, or spindle- or dumbbell-shaped, with conspicuous and prominent eyes that are evenly distributed (Pfannenstiel and Slack 1980). In many cases, PSTVd cannot be diagnosed reliably by inspecting potato plants because symptoms are often indistinct or absent (Sing et al. 1970).

Considerable progress has been made during the past several years in establishing the primary and secondary structures of viroids. The complete nt primary sequence of PSTVd has been determined, and the covalently closed circular RNA forms an extended rod-like structure characterized by a series of double helical sections and internal loops (Gross et al. 1978; Owens and Cress 1980).

Although the first cases of PSTVd in Turkey were recorded in ornamental plants (Verhoeven et al. 2009), potatoes (Önelge and Bozan 2005; Bostan et al. 2010), and tomatoes (Bostan et al. 2010), no molecular characterization of this agent has been officially reported from potato. Here, we carry out a survey of several commercial potato tuber sellers in Turkey for the incidence of PSTVd and we report some biological and molecular properties of a PSTVd isolate detected from a commercial tuber variety.

Materials and methods

Source of positive control

The PSTVd potato isolate used as the positive control in this study was obtained from the Plant Protection Service of Wageningen, the Netherlands, with the permission of the General Directorate of Plant Protection and Control of the Turkish Ministry of Agriculture and Rural Affairs in Ankara. The viroid was maintained in a tomato plant following mechanical inoculation in a growth chamber.

Biological characterization

The selected isolate was named PSTVd-TR, from Turkey. PSTVd-TR-infected eye samples were collected from individual potato tubers. Leaves of tomato seedlings (cultivar Joker) were sap-inoculated by rubbing with an extract prepared in 0.1 M sodium phosphate (pH 7.2) containing a small quantity of carborundum dust ground together with infected eyes of seedling tubers. Noninoculated plants of the same species were included as controls. Test plants were kept under observation for 10 weeks after inoculation. Samples collected from the uppermost

leaves 4-5 weeks after inoculation were analyzed for the presence of PSTVd by reverse transcriptase polymerase chain reaction (RT-PCR) using genome specific primers.

Collecting tubers

Commercial sellers are well-established potato tuber distributors throughout Turkey and serve more than 70% of the country. In January and February of 2010, 168 seedling tubers of *Solanum tuberosum* L. were randomly collected from 17 different commercial sellers in Turkey.

RNA extraction, reverse transcription and RT-PCR

RNA extraction was carried out according to the silica-capture method from seedling tubers as described by Foissac et al. (2001). The RT-PCR assay was carried out in a 2-step procedure as described by Sipahioglu et al. (2006). In the detection of PSTVd isolates, a pair of genome specific primer sets (P-sense 5'-ACTCGTGGTTCCTGTGGTTC-3' and P-antisense 5'-TGTTTCCACCGGGTAGTAGC-3') was used for generating a 264-bp genome fragment (Singh 1999).

Primer design, construction of a PSTVd-specific clone and *in vitro* transcription of digoxigenin-labeled RNA probe

The PSTVd-TR genome was generated by RT-PCR by using the end-to-end primers (P-*Hind* III-F 5'-*CAGT*AAGCTTCGGAACTAAACTCGTGGTTCC-3', P-*Pst*I-R 5'-*CAGT*CTGCAGAGGAACCAACTGCGGTTCCA-3') designed to encompass the unique endonuclease restriction sites *Hind*III and *Pst*I (underlined) and 4 additional unrelated residues at their 5' end (italicized). The PCR products were subsequently inserted into the pSPT18 vector (Roche Inc., USA) and transformed into *Escherichia coli* (MJ 109) by electroporation (Bio-Rad, USA). Following the purification of recombinant plasmids, the restriction digest was performed in order to generate DIG-labeled RNA probes. These clones were digested with *Hind*III or *Pst*I and transcribed with SP6 or T7 RNA polymerase, according to manufacturer instructions (Roche Inc.; Cat. No. 11175025910) in the presence of DIG-11-UTP to obtain digoxigenin (DIG)-labeled viral riboprobes of plus and minus polarities, respectively. The transcripts of PSTVd subgenomic cDNA inserted into cloning vector pSPT18 were used as probes in molecular hybridizations with viroid RNA.

Molecular hybridization

RNA extracts from potato tissue were processed and applied to a nylon membrane using a dot blot apparatus (Scie-Plas Ltd., UK). The nucleic acids were further bound by ultraviolet light exposure for 3 min. Prehybridization was carried out for 2 h at 56 °C in 50% deionized formamide, 5X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% (w/v) N-laourylsarcosine, 0.02% (w/v) SDS, and 5% (w/v) blocking reagent (Roche Inc.). The prehybridization solution was removed and replaced with 20 mL of hybridization mixture containing a DIG-labeled RNA probe denatured by heating at 70 °C for 2 min; it was then incubated overnight at 56 °C. After hybridization, the membrane was washed twice in 2X SSC containing 0.1% SDS at room temperature for 5 min and twice in 0.1X SSC containing 0.1% SDS at 68 °C for 15 min. To detect the hybridized probe, binding to anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche Inc.) and subsequent chemiluminescent detection using CSPD (disodium 3-(4-methoxyspiro {1,2-dioxtane 3,2'-(5 chloro) tricyclo[3.3.1.1^{3,7}]decan}4-yl) phenyl phosphate) (Roche Inc.; Cat. No. 11363514910) as the substrate were used (Pallas et al. 1998).

Cloning and sequencing of complete genome

Full-length double-stranded cDNA was separated on a 2% agarose gel, recovered using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), and purified with the DNA Clean and Concentrator™ Kit (Zymo Research) according to manufacturer instructions. The purified DNA fragments were ligated into the pSPT18 vector to transform into *Escherichia coli* MJ 109, as described above. The cDNA clones were sequenced using an automated DNA sequencer (Applied Biosystems, USA).

Prediction of secondary structure

In order to predict the most stable secondary structure of the PSTVd-TR isolate, computer analysis was performed on the established full sequence. For this purpose, the mfold structure prediction package of CLC RNA Workbench Version 4.4 (CLC bio, Denmark) was used.

Determination of sequence homology and phylogenetic analysis

For the sequence similarity and phylogenetic analysis, the following GenBank accessions for PSTVd sequences from different hosts were used: GU481091, EF192393, and FM998542 from *Solanum jasminoides*; EF459700 from *Solanum rantonnetii*; FJ872823 from *Lycopersicon esculentum*; and V01465 from *Solanum tuberosum*. The whole sequence of the PSTVd-TR isolate (GenBank Accession No. HQ456944) was compared using the Vector NTI software program (Invitrogen, USA).

Results

The incidence of PSTVd infection in the tested samples

To test whether the synthesized plus and minus polarity riboprobes were suitable for routine diagnosis of PSTVd, dot blot molecular hybridization experiments were carried out with DIG-labeled RNA probes. A single hybridization signal was detected in RNAs from PSTVd-infected tissue. The highest intensity was obtained when RNA preparations were probed with PSTVd riboprobes of minus polarity. Thus, the minus polarity riboprobes of PSTVd were used for diagnostic purposes.

PSTVd was detected in 4% of the samples tested in 2010 (Table 1). Only 1 potato sample (cultivar Russet Burbank) reacted positively for PSTVd in the molecular hybridization (MH) tests (Figure 1). No hybridization signals were observed in the total RNA extracted from the healthy control plant (Figure 1). In MH, 6 samples gave weaker hybridization signals than unique positives, and all of those samples were considered suspicious. The same nucleic acid preparations of suspicious samples analyzed previously by dot blot hybridization were used as templates in RT-PCR experiments. All suspected samples reacted positively in RT-PCR. A 264-bp amplicon was generated from all suspected potato samples (cultivars Innovator, Lady Jo, Russet Burbank, Agria, Provento, and Konsül) using genome specific primers (P-sense/P-antisense); no fragment was observed in the extract from a healthy potato tuber (Figure 2). The specificity of amplified cDNAs was confirmed by cloning and sequencing one of the amplified products.

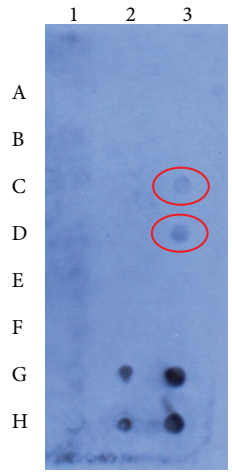

Figure 1. Detection of PSTVd with dot blot hybridization by DIG-labeled RNA probes. Columns A1-H1, A2-F2, and A3-B3 contain the potato extracts tested against PSTVd. E3 and F3 are negative controls, G3 and H3 are positive controls obtained from the Netherlands, G2 and H2 are commercial kit positives, and C3 and D3 are positive samples detected in MH testing as 2 repetitions. Circled samples are those selected for cloning and sequence analysis.

Complete nucleotide sequence and most stable secondary structure of PSTVd-TR

The complete double-stranded cDNA of the selected PSTVd isolate (PSTVd-TR) was cloned and sequenced in the pSPT18 vector by using a pair of end-specific primers. The RNA of the covalently closed, circular, single-stranded PSTVd-TR isolate consists of 358 nts. BLAST analysis of the sequenced full genome (GenBank Accession No. HQ456944) revealed that the highest identity (99%) was with a PSTVd isolate recently identified in *Solanum rantonnetii* in Italy (Accession No. EF459700) (Table 2 and Figure 3).

The primary sequence analysis of the PSTVd-TR isolate revealed insertions, deletions, and substitutions of nts at different sites of the PSTVd-TR RNA molecule (Figure 3). As compared with the PSTVd type strain (PSTVd, V01465), they are located at nt positions 64, 127-238, and 121-124-126-299-308.

In order to predict the most stable secondary structure of the PSTVd-TR isolate, we performed computer analysis on the established full sequence. We used the mfold structure prediction package of CLC RNA Workbench Version 4.4. A prediction of

Table 1. Results of series of tests conducted to verify the presence of PSTVd in selected commercial potato cultivars. Samples tested by MH or RT-PCR are indicated by "+."

Cultivar	Number of samples tested	Tests applied to seedling potato tubers		Number of samples infected	Overall infection rate (%)
		MH	PCR		
Opal	5	+	-	-	
Lady Rosetta	7	+	-	-	
Satina	2	+	-	-	
Marabel	9	+	-	-	
Agria	22	+	+	2	
Lady Claire	15	+	-	-	
Konsül	11	+	+	1	
Ramos	3	+	-	-	
Cycloon	1	+	-	-	
Hermes	21	+	-	-	
Russet Burbank	10	+	+	1	
Marfona	14	+	-	-	
Lady Olympia	8	+	-	-	
Fabulo	1	+	-	-	4
Granola	5	+	-	-	
Victoria	2	+	-	-	
Innovator	2	+	+	1	
Binella	2	+	-	-	
Slaney	1	+	-	-	
Adora	3	+	-	-	
Lady Jo	5	+	+	1	
Shepody	1	+	-	-	
Provento	5	+	+	1	
Agata	5	+	-	-	
Sante	4	+	-	-	
Safrane	1	+	-	-	
Verdi	3	+	-	-	
Total	168			7	

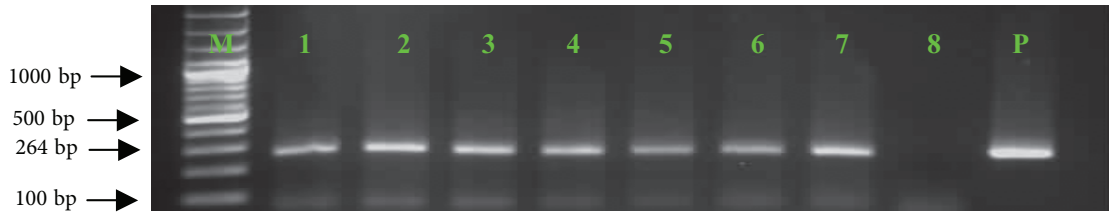

Figure 2. RT-PCR testing revealed the presence of PSTVd infections in suspicious samples from MH testing. 1-6: suspected samples in MH, 7: positive sample detected in MH (cultivar Russet Burbank), 8: healthy potato tuber, P: positive control from the Netherlands, M: 3000-bp molecular size markers (Fermentas, Lithuania).

Table 2. Designation, geographic origin and GenBank accession numbers of PSTVd-TR isolate and other viroid isolates used for comparison of nucleotide sequences.

Isolate name	Country	GenBank accession no.	Genome size	Host
PSTVd KalV66	Greece	GU481091	357	*Solanum jasminoides*
3373056	The Netherlands	EF192393	357	*Solanum jasminoides*
PS-Sr	Italy 1	EF459700	357	*Solanum rantonnetii*
PSTVd-ToIt	Italy 2	FJ872823	357	*Lycopersicon esculentum*
06094505	Belgium	FM998542	357	*Solanum jasminoides*
Type strain	Germany	V01465	359	*Solanum tuberosum*
PSTVd-TR	Turkey	HQ456944	358	*Solanum tuberosum*

the secondary structure was obtained. As expected, the PSTVd-TR isolate adopted a basic rod-like most stable secondary structure (Figure 4a). The virulence modulating region (VMR) was very conserved compared to the 7 isolates that it was measured against. No nt differences were detected between PSTVd-TR and the other world isolates in the VMR domain of the viroids (Figure 4b). The structure predictions of all compared viroids showed no particular tendency toward branched secondary structures. Secondary structure analysis of these sequences (Figure 4c) clearly demonstrated that the 8 nt differences between PSTVd-TR and the PSTVd type strain in the left- and right-hand regions affect the most stable secondary structure only locally.

Transmission studies

For the infectivity assays, 5 viroid-free plants of tomato cultivar Joker were inoculated with an extract obtained from a PSTVd-TR-infected *S. tuberosum* tuber. Transfer of the PSTVd-TR isolate to tomato produced a symptomless infection. Strong positive reactions were observed when samples of silica-clarified RNA prepared from infected tomato plants were analyzed by RT-PCR using genome-specific primers (data not shown).

Discussion

It was evident from survey results that disease incidence was low (4%) in seedling potato tubers delivered by commercial sellers in Turkey. Thus, our findings agree with those of Önelge and Bozan (2005) and Bostan (2010), who observed a low incidence of PSTVd in potato tubers (approximately 1%-2%). This was the first report of a survey of commercial sellers aimed at determining the incidence of PSTVd in potato tubers, although preliminary field surveys for potato plants in Turkey were reported in 2005 and 2010 (Önelge and Bozan 2005; Bostan 2010).

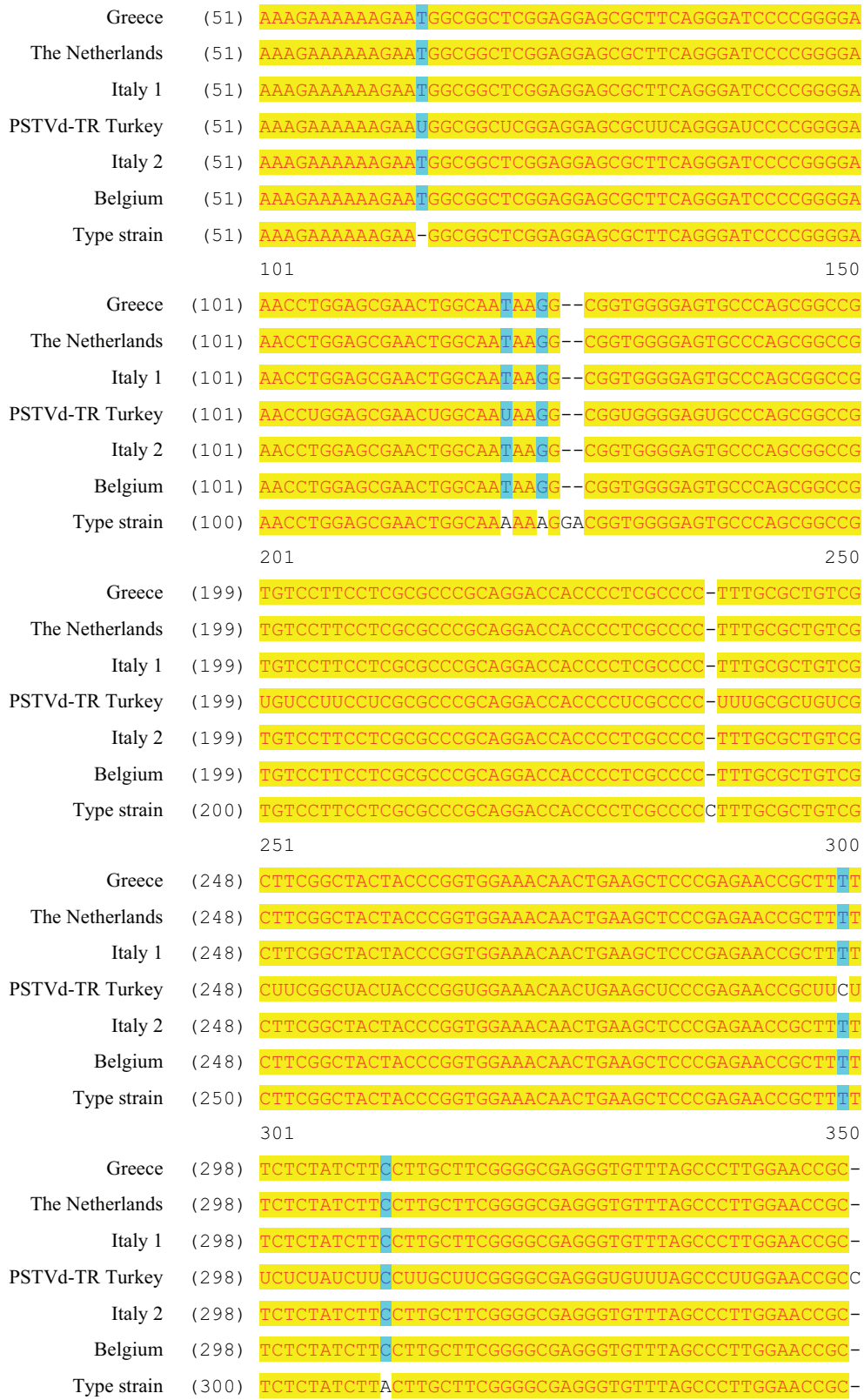

Figure 3. Comparison of the nucleotide sequence of PSTVd isolates as shown in the aligned primary structure of their RNA. Only those regions in which the sequence of the isolates differs are given. The shadowed areas represent the regions of nucleotide homology.

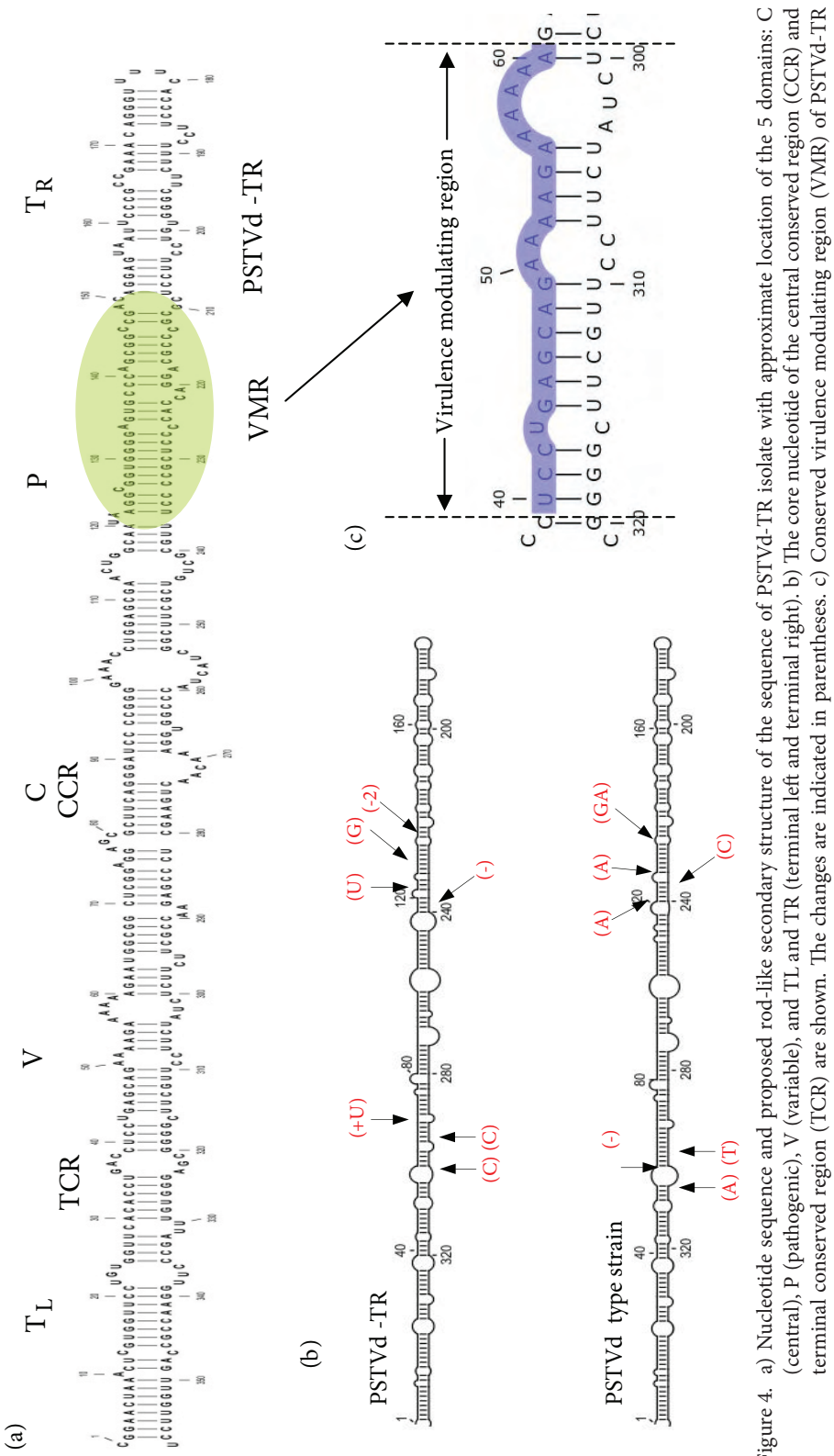

Figure 4. a) Nucleotide sequence and proposed rod-like secondary structure of the sequence of PSTVd-TR isolate with approximate location of the 5 domains: C (central), P (pathogenic), V (variable), and TL and TR (terminal left and terminal right). b) The core nucleotide of the central conserved region (CCR) and terminal conserved region (TCR) are shown. The changes are indicated in parentheses. c) Conserved virulence modulating region (VMR) of PSTVd-TR isolate.

Only one sample reacted positively in the MH tests. The same sample and 6 other suspicious samples were detected as positive when they were assayed by RT-PCR. Dot blot hybridization assays on RNA extracts using a DIG-labeled riboprobe failed to detect the suspected viroid infections, possibly because of the low concentration of target RNA. Inconsistent molecular hybridization and RT-PCR test results have been reported previously (Sanchez-Navarro et al. 1998). Sanchez-Navarro et al. (1998) revealed that the nonradioactive dot blot hybridization method was 625 times less sensitive than the RT-PCR technique. Although the PCR method is more sensitive than the dot blot hybridization method for viroid detection, its use in large scale surveys may not be reasonable because of the cost and the time-consuming manipulations. Nevertheless, PCR assays are essential in the framework of sanitary improvement programs for checking results of molecular hybridization tests whenever there is a negative or suspected response (Alabdullah et al. 2009).

Molecular cloning revealed that the PSTVd-TR isolate consists of 358 nts (Figure 4a). In potato it is commonly 359 nts in length (Gross et al. 1978), and more rarely 358 and 360 nts (Herold et al. 1992; Lakshman and Tavantzis 1993). Except for 1 isolate of 341 nts (Wassenegger et al. 1994), such isolates are very similar in size and vary by 1-7 nts from the prototype strain (Gross et al. 1978; Singh et al. 1999). Lengths of 356 nts have been found in wild *Solanum* spp. (Behjatnia et al. 1996) and tomato (*Lycopersicon esculentum*) (Puchta et al. 1990). Lengths of 356 and 357 nts have been reported in pepino (*S. muricatum*) (Puchta et al. 1990; Shamloul et al. 1997).

We compared the sequence characterized in this study to those already known from the databases. Our novel sequences showed only minor modifications compared to those described previously. Comparison of the nt sequences of PSTVd-TR revealed a high sequence similarity with the PSTVd type strain and the other PSTVd isolates reported from different geographical regions and hosts. The results suggest that there is no clear relationship among the host specificity and geographical origin of PSTVd isolates.

The main characteristic of viroids is their infectivity, i.e. their ability to initiate replication and the production of viroid progeny when inoculated onto suitable host plants (Diener 1987; Semancik 1987). Infected tomato plants developed no characteristic macroscopic symptoms such as those described by Di Serio (2007) for PSTVd. A BLAST search using the NCBI server revealed that the sequences closest to the new PSTVd-TR isolate reported in this work are those from Europe, which have the ability to produce stunting and darkening on potato leaves (Di Serio 2007).

The secondary structures of the isolate predicted using the mfold software showed that the most stable secondary structure of PSTVd-TR was a classical rod-like structure with no additional hairpins. The master sequences of all PSTVd isolates, including the TR isolate, were able to fold into a characteristic rod-like secondary structure in which around 75% of the nts were paired. With time, the rod-like secondary structure has almost become an identification criterion for viroids (Bussière et al. 1996). Most of the published viroid secondary structures were predicted with the original RNA fold package. These original predictions led to the proposal that the most stable secondary structure of the classical viroids of the PSTVd-type (i.e. PSTVd and apple scar skin group viroid groups) are rod-like structures composed of alternating single- and double-stranded regions (Riesner and Gross 1985; Diener 1993). The results clearly indicated that all of the isolates may fold as a rod-shaped secondary structure as a result of the complete reorganization of the base pairing observed in the minimum free energy rod-like structure. However, it remains unknown whether these predicted structures are adopted in solution or whether they serve a particular function in vivo (Bussière et al. 1996).

When comparing the VMR of PSTVd-TR, 20 nts within a sequence element are identical. Sequence variants of PSTVd with structural differences in the VMR were identified as naturally occurring (Lakshman and Tavantzis 1993) and were demonstrated to be responsible for differences in symptom induction and sequence stability (Gandía et al. 2000)

Until this study was initiated, PSTVd, a disease of quarantine status in Turkey, was believed to be absent from commercial potato production in Turkey. Our data provide adequate proof that PSTVd is present in the Turkish commercial potato industry. Although

our results clearly indicated that the viroid was present throughout Turkey (Table 1), the actual incidence and distribution could not be deduced from the data due to the irregular number of samples tested from commercial sellers. The full sequence of the PSTVd-TR from Turkey was similar to those of European isolates, suggesting genetic stability in the agent.

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