

Full Length Research Paper

Occurrence of *Pospiviroid* in potato, tomato and some ornamental plants in Turkey

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In order to investigate the *Pospiviroid* in potato, tomato and some ornamental plants in Turkey, a survey study was carried out from 2006 to 2009. During this survey study, a total of 1766 samples (1047 potato samples, 258 tomato leaves and 461 ornamental plants) were tested by reverse transcription-polymerase chain reaction (RT-PCR) and, the samples found to be positive in RT-PCR were also checked in return polyacrylamide gel electrophoresis (R-PAGE). Of these samples, potato tubers were collected from markets, leaf samples from potato fields (Erzurum), tomato samples from field (Tokat, Amasya, Balıkesir and Bursa) and greenhouses (Antalya and Muğla), leaves and vines of ornamental plants from greenhouses (Yalova) and florists (Erzurum) and recreation areas (Ankara, İzmir and Trabzon). In the result of RT-PCR test, PSTVd was detected in 6 out of 891 potato tubers and CSVd in 2 out of 154 chrysanthemums. On the other side, tomato, citrus and most ornamental plants belonging to different families were found to be free from *pospiviroid*. This is the first report of CSVd in Turkey.

Key words: Potato spindle tuber viroid, chrysanthemum stunt viroid, potato, Chrysanthemum, viroid, Turkey.

INTRODUCTION

Viroids are the smallest infectious agents, circular single-stranded RNA molecules, consisting of 239-475 nucleotides (nt) and infect many economically important horticultural and agricultural crops (Diener, 2001; Flores et al., 2005; Tsagris et al., 2008). The symptoms caused

by viroids are generally similar to those caused by viruses in higher plants. Depending on the host plant and viroid species, expression of infection may vary from symptomless to severe stunting, leaf or stem necrosis, flowering alterations and foliar or fruit deformation (Singh et al., 2003; Daros et al., 2006). Like viruses, viroids are transmitted by mechanical wounding and infect their hosts systemically. Although viroids resemble viruses in certain aspects (example, the type of symptoms that they induce in their hosts) they also differ in fundamental aspects that include structure and function (Flores et al., 2003; Singh et al., 2003). However, unlike plant viruses, these plant pathogenic RNAs lack the capacity to code for proteins and, therefore, the viroid life cycle is strictly dependent on host factors (Steger and Riesner, 2003).

Most of the 30 known viroid species affecting agricultural and horticultural plants have been recognized because they induce disease symptoms in their host plants (Flores et al., 2003). On the basis of biochemical, biological and

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Abbreviations: RT-PCR, Reverse transcription-polymerase chain reaction; R-PAGE, return polyacrylamide gel electrophoresis; PSTVd, potato spindle tuber viroid; TCDVd, tomato chlorotic dwarf viroid; MPVd, Mexican papito viroid; TPMVd, tomato planta macho viroid; CSVd, chrysanthemum stunt viroid; CEVd, citrus exocortis viroid; TASVd, tomato apical stunt viroid; IrVd, iresine viroid; CLVd, columnea latent viroid; CVd, citrus viroids; RT, reverse transcription; GYSVd, Grapevine yellow speckle viroid; HSVd, hop stunt viroid; PLMVd, peach latent mosaic viroid; CChMVd, chrysanthemum chlorotic mottle viroid.

structural properties, have been classified into two families, the *Pospiviroidae*, which replicate in the nucleus and the *Avsunviroidae*, which replicate (and accumulate) in the chloroplast (Flores et al., 2003; Steger and Riesner, 2003; Flores et al., 2005; Tsagris et al., 2008). Most of viroids belong to the family *Pospiviroidae* which is containing five genera (*Pospiviroid*, *Hostuviroid*, *Cocadviroid*, *Apiscaviroid* and *Coleoviroid*). Within these genera, the genus *Pospiviroid* has nine viroid species. They are *Potato spindle tuber viroid* (PSTVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Mexican papito viroid* (MPVd), *Tomato planta macho viroid* (TPMVd), *Chrysan-themum stunt viroid* (CSVd), *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd), *Iresine viroid* (IrVd) and *Columnnea latent viroid* (CLVd) (Hadidi and Candresse 2003).

Viroids cannot be detected by immunological methods, because they do not encode any proteins (Steger and Riesner, 2003). Therefore, an accurate and reliable diagnostic method for detection of viroids and followed by eradication of infected sources is needed for quarantine operations. Several diagnostic methods have been used for the detection of viroids. However, molecular techniques (reverse transcription polymerase chain reaction (RT-PCR) and nucleic acid hybridization) are considered reliable, fast and sensitive detection techniques and could be applied to the large-scale and multiple detection of plant pathogens (Ito et al., 2002; Cohen et al., 2006).

In previous studies, the presence of *Citrus viroids* (CVd-IIIa and CVd-IV) in Citrus lemon (Onelge et al., 2000), *Grapevine yellow speckle viroid* (GYSVd-1 and GYSVd-1) and *Citrus exocortis viroid-grapevine* (CEVd-g) in Grapevine (Gazel and Onelge, 2003), *Hop stunt viroid* (HSVd) in Grapevine (Gazel and Onelge, 2003; Gazel et al., 2008) and in apricot (Sipahioglu et al., 2002 and Sipahioglu et al., 2006), PSTVd in potato (Onelge and Bozan, 2005) and *Peach latent mosaic viroid* (PLMVd) in stone fruit (Gümüs et al., 2007) was reported in Turkey. However, it was not any research report about *Pospiviroid* potato, tomato and ornamental plants in Turkey.

The objective of this survey study was to investigate of the genus *Pospiviroid* in potato, tomato and some ornamental plants using RT-PCR and return polyacrylamide gel electrophoresis (R-PAGE).

MATERIALS AND METHODS

Plant samples

This study was carried out during the years of 2006 and 2009. Potato tubers showing symptoms peculiar to PSTVd (spindly-shaped tubers, abnormally shaped tubers and cracks tubers) were collected from markets (Erzurum), tomato samples from field (Tokat, Amasya, Balıkesir and Bursa) and greenhouses (Antalya and Muğla), chrysanthemum samples from nurseries (Yalova) and florist (Erzurum). Other annual and perennial ornamental plant samples were collected from nurseries (Erzurum) and recreation areas (Ankara, İzmir and Bursa). PSTVd control was kindly supplied by R.P. Singh (Potato Research Centre, Agriculture and Agri-Food Canada, Fredericton, NB, Canada).

Nucleic acid isolation

Nucleic acids were isolated from potato tubers and the plant leaves, as described previously (Singh and Boucher, 1987) with some modifications. In brief, 300 µl extraction buffer [0.53 M NH₄OH, 0.013 M disodium ethylenediaminetetraacetate (EDTA) pH 7.0, 4M LiCl] and 400 µl of Tris-saturated phenol (containing 0.1 g of 8-hydroxyquinoline per 100 ml) was combined with five drops (200 µl) of sap. The sap was obtained from tuber or leaves tissues by a Tuber Slicer (Electrowerk, Behcke and Co., Hannover, Germany). The suspension was centrifuged (15 min, 12,000 × g) at 4°C and the nucleic acids were precipitated from the aqueous layer with 2.5 vol of ethanol in the presence of 0.1 vol of 4 M sodium acetate (-20°C, overnight). The precipitate was collected by centrifugation (12 000 × g, 15 min, 4°C), washed with 70% ethanol, vacuum-dried and dissolved in 200 µl (leaf sample) or 100 µl (tuber sample) of d₂HO for RT-PCR and R-PAGE.

Reverse transcription-polymerase chain reaction (RT-PCR)

For the reverse transcription (RT), nucleic acid extract (2.5 µl) was incubated at 65°C for 5 min and then chilled on ice for 10 min to denature the RNA. The RT mixture of 7.5 µl was added to provide a final concentration of 20 ng/µl of reverse primer, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DDT), 2.5 mM MgCl₂, 1.5 mM of each dNTPs (Promega), 200 U RNasin (Promega) and 20 U Moloney Murine Leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). Samples were incubated for 1 h at 42°C for RT and subsequently incubated at 95°C for 3 min to terminate the reaction.

PCR was performed using 2 µl of cDNA mixture in 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5mM MgCl₂, 200 mM each of dNTPs [dATP, dCTP, dGTP and dTTP (Promega)], 0.1 mM each of the primers and 0.625 U of DNA polymerase (Sigma). Thermo-cycling was performed as described previously (Bostan et al., 2004). Samples were amplified in 35 cycles. Annealing temperature was 62°C (30 s) denaturation (92°C, 30 s) and primer extension (72°C, 90 s), and a final extension (72°C, 10 min). Ten microliters of amplified products were separated by electrophoresis in a 2.0% agarose gel containing 0.5 µg/ml ethidium bromides and photographed. For RT-PCR, the reverse (5'-AGC TTC AGT TGT TTC CAC CGG GT-3') and forward (5'-ATT AAT CCC CGG GGA AAC CTG GAG-3') primer pairs designed from conserved region of the members of genus *Pospiviroid* (Bostan et al., 2004) were used.

Return-polyacrylamide gel electrophoresis (R-PAGE)

In the R-PAGE assay, nucleic acids are subjected to two electrophoretic runs, one under non-denaturing and the other denaturing conditions as described (Singh and Boucher, 1987). Briefly, 40% of glycerol and 10 µl of a solution consisting of 1% of xylene cyanol FF and 1% bromophenol blue was added to the nucleic acids dissolved in the 'high salt' 1 × TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH: 8.3). First, electrophoresis separation of the nucleic acids was at constant current 46 mA for 2.5 h using an SE 600 series apparatus (Hoefer Scientific Instruments, San Francisco, CA) on 5% non-denaturing slab gels (5% polyacrylamide, 0.125% bis-acrylamide, 14 cm × 16 cm × 0.15 cm) in the high salt buffer, using 10 µl of sample in each well. For the second run, the buffer in both the upper and lower reservoirs was replaced with a 'low salt' buffer (1: 8 dilution of the high salt buffer). About 2 L of the low salt buffer heated to boiling was poured into the lower electrophoresis chamber in which the first gel was immersed. These conditions denatured the viroid. The remainder of the lower electrophoresis chamber was filled with 3 L of buffer heated to 73-75°C. The polarity was reversed and the electrophoresis was performed at 75°C (46 mA constant current, 2 h). Gels were stained using silver nitrate and photographed.



Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) detection of two *Pospiviroids* from potato tubers and chrysanthemum. Lanes 1, 2, 3, 4, 5 and 6 PSTVd isolates; lane 7, healthy potato tuber (negative control); lane 8, positive control for PSTVd; lane 8, healthy Chrysanthemum and lane 10, CSVd from chrysanthemum).

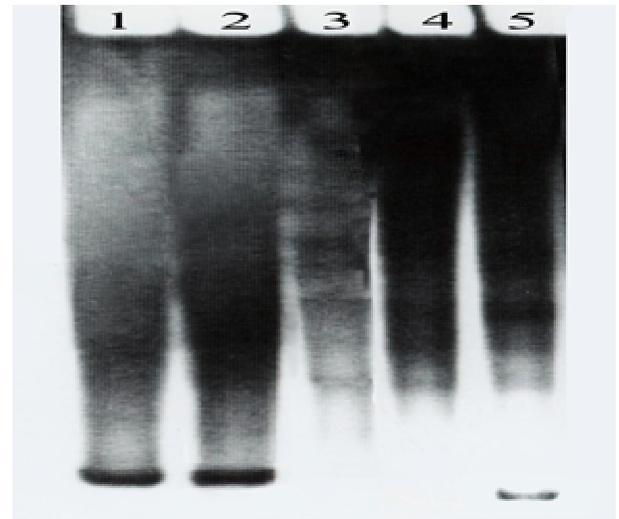


Figure 2. Return-polyacrylamide gel electrophoresis (R-PAGE) detection of two viroids. Lane 1, PSTVd positive control (potato tuber); lane 2, PSTVd sample from potato tuber; lane 3, healthy potato tuber; lane 4, healthy chrysanthemum and lanes 5, CSVd from chrysanthemum.

RESULTS AND DISCUSSION

During this survey study, a total of 1766 samples (891 potato tubers, 156 potato leaves, 258 tomato leaves and 461 ornamental plants) were tested with RT-PCR. Of these samples, potato tubers were collected from markets, leaf samples from potato fields (Erzurum), tomato samples from field (Tokat, Amasya, Balıkesir and Bursa) and greenhouses (Antalya and Muğla), chrysanthemum samples from greenhouses (Yalova) and florists (Erzurum). The leaves and vines of the other ornamental plant samples were collected from nurseries (Erzurum) and recreation areas (Ankara, İzmir, Bursa).

In the result of RT-PCR test, PSTVd was detected in 4 out of 891 potato tubers and CSVd in 2 out of 154 chrysanthemums collected from greenhouses in Yalova. No amplification fragment was observed in healthy and negative controls in RT-PCR (Figure 1). On the other side, tomato and most ornamental plants belonging to different families was found to be free from *Pospiviroid*. According to the result, it can be said that the incidence rate of these viroids in the tested samples is considerably low in Turkey. PSTVd was previously detected in potato tubers with low incidence (1.2 - 1.8%) in Turkey (Onelge and Bozan, 2005). CSVd was reported for the first time in Turkey. Actually, three viroids infects chrysanthemums, however our primer can detect only CSVd. There are two main viroids that infect chrysanthemum: CSVd (*Pospiviroidae*) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) (*Avsunviroidae*) (Hadidi and Candresse 2003). But primer pairs used in this study was only specific to the genera of *Pospiviroid*.

Expected fragment sizes in RT-PCR were 199 bp for both PSTVd and CSVd. The forward primer nt 89-108 in the upper central conserved region (CCR) and the reverse primer nt 259-280 from the lower CCR in PSTVd. Therefore, all *Pospiviroids* except *Columnnea latent viroid* (CLVd) were expected to be detected if present in the plant samples (Bostan et al., 2004). RT-PCR positive samples were also checked to detect the viroid molecules and the location of band in R-PAGE (Figure 2).

Expected band location from PSTVd and CSVd in R-PAGE was near at each other. PSTVd and CSVd have a genome size 354, 359 nt, respectively. However, electrophoretic mobility of PSTVd band from sample was 2-3 mm slower than CSVd in R-PAGE. Bands are easily observed in infected samples using both RT-PCR and R-PAGE whereas they were not observed in healthy samples. These result shows that the samples were infected by viroids.

The type species of the genus, *Potato spindle tuber viroid* (PSTVd), infects potato and other solanaceae and nonsolanaceae plants (Singh et al., 1999, 2003) and it is readily transmitted mechanically and, in the case of potato or tomato, is also transmitted through both botanical seed and pollen (Singh et al., 1992). Furthermore, PSTVd and PLRV can be transmitted by the aphid *Myzus persicae* in a persistent manner (Puchta et al., 1990; Salazar et al., 2001; Owens, 2007). In addition, potatoes, PSTVd was reported in tomato (Elliott et al. 2001; Mumford et al., 2004; Verhoeven et al., 2007c), in capsicum (pepper) (Lebas et al., 2005), in avocado (*Persea americana*) (Querici et al., 1995), in pepino (*Solanum muricatum*) (Puchta et al., 1990; Shamloul et al., 1997) and several ornamental plants belonging to the

family *Solanaceae* (Di Serio, 2007; Verhoeven et al., 2007a, b).

The source of infection is uncertain, because, there are not any official and private seed potato companies, producing pre-basic seeds, in Turkey and sophisticated certification laboratory where imported seed potato seeds will be tested as well. Hence, to meet the demand of farmers, seed potatoes have been imported from other countries (mainly European) as pre-basic, then multiplied and distributed to the producers by some private companies. It is therefore possible that the origin of PSTVd infections on potatoes in Turkey is either from Turkey or seed-exporting countries. PSTVd may have been introduced in the past. The comment is justified by the fact that, in spite of reliable test procedures for PSTVd detection, viroid is still escaping detection in originally accession. On the other hand, in Turkey, whole tubers are planted, minimizing the transmission associated with cutting tools and multiplication by cut pieces. Also, the introduction of PSTVd from imported seed may not build up to severe level because potato crops planted with imported seed usually last 3 - 4 years and then a new seed source is used for planting.

The main hosts of CSVd are florists chrysanthemums (*Dendranthema morifolium*) and related ornamentals including *Chrysanthemum prealtum*, *D. indicum* and *Tanacetum parthenium*. Spread of CSVd is most unlikely by natural means, but can result from transplant of infected chrysanthemum plants and cuttings; there is also the possibility that the viroid is present in plant material of other species. Control of the disease is extremely difficult due to its highly contagious nature and long latent period.

As potato seed, several economically important ornamental plants and their hybrids are imported as either pre-basic or seed from various countries, propagated and then marketed either as cutting flowers or ornamental plants. Majority of viroids are known to spread in nurseries and greenhouses by the use of viroid contaminated tools (Singh, 1983; Bostan et al., 2004). Furthermore, several *Pospiviroid* were transmitted via various ornamental plants seed (Singh et al., 2009). Therefore, ornamental plants have been considered an excellent viroid reservoir (Hammond et al., 1989; Singh et al., 1992). In this case, the source of CSVd may be different plants and different countries. Several *Pospiviroid* have been identified and isolated from various ornamental plants (Hammond et al., 1989; Singh et al., 1991, 1992; Spieker 1996; Spieker et al., 1996).

As a result, it is obvious that a sophisticated certification laboratory is urgently needed in Turkey, for early detection is the main way to the control of virus and viroid diseases.

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