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Nucleotide Sequence and Secondary Structure of *Potato Spindle Tuber Viroid* Egyptian Strain

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ABSTRACT

Potato Spindle Tuber Viroid (PSTVd-$_{EG}$) was isolated from naturally infected potato plants cv. Diamond during autumn season. A reverse transcription-polymerase chain reaction (RT-PCR) was used for PSTVd identification using specific primer of Central Conserved Region (CCR). A major expected band with approximately 359 bp was detected in total nucleic acid extracted from PSTVd infected tissues. Southern- and dot-blot hybridizations were used to confirm the PCR- product. The 359 bp amplified c-DNA was hybridized with Digoxigenin (DIG)-labeled PSTVd-cDNA. The partial nucleotide sequence of PSTVd-$_{EG}$ strain was determined and compared with five geographical distinct PSTVd-$_{EG}$ strains available from GenBank and the results revealed to presence of high sequence identity (89-84 %). Secondary structure was confirmed for PSTVd-$_{EG}$ strain with minimum free energy of -116.51 kcal /mol. Partial nucleotide sequence of PSTVd-$_{EG}$ was recorded in GenBank with accession number EU273604.

Key Words: Potato, PSTVd, RT-PCR, nucleic acid hybridization, nucleotide sequencing, secondary structure.

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INTRODUCTION

Potato Spindle Tuber Viroid (PSTVd) is the type member of the genus *Pospiviroid* (Family *Pospiviroidae*). It is a single-stranded, circular RNA molecule with size length of, about 356-361 nt. and non-encapsidated *(Schnöelzer et al., 1985)*. It consists of five structural domains central conserved region (CCR), pathogenic (P), variable (V) and Left and Right terminal domains (T_1 and T_2). Due to a high degree of internal sequence complementarities, PSTVd RNA has a complex secondary structure *(Kalantidis et al., 2007)*. The secondary structure of PSTVd during thermal denaturation undergo several structural transitions from the rod-like structure to the single stranded circle without any intramolecular base pairing *(Loss et al., 1991)*. In a highly cooperative main transition all base pairs of the native structure are disrupted and particularly stable hairpins, newly formed (Hp I, Hp II, Hp III). Secondary structure almost became an identification criterion for viroids *(Bussier et al., 1996)*. In addition, *Mathews* and *Turner (2006)* observed that the best known algorithms for predicting the secondary structure of a single RNA molecule by finding the minimum free energy (mfe) which based on dynamic programming algorithms. Serra, et al. *(1995)* assume that the total free energy of a given secondary structure for a molecule is the sum of independent contributions of adjacent, or stacked, base pairs in stems (which tend to stabilize the structure) and of loops (which tend to destabilize the structure). The aim of this work illustrated the primary and secondary structure of *Potato Spindle Tuber Viroid* Egyptian strain (PSTVd-$_{EG}$).

MATERIALS AND METHODS

Plant Materials:

Tuber potato samples *(Solanum tuberosum* L.*)* cv. Diamond were collected from Menoufia governorate, Center International Potato (CIP) and Kafer El-Zeite at autumn season. These tubers exhibited PSTVd-like symptoms, spindle, long, cylindrical narrow shapes, smooth skinned and the eyes are conspicuous as well as, tuber infected with PSTVd- positive control (Virology Lab. Fac of Agric. Ain Shams Univ *(El-Dougdoug, 1988))*.

Dot Blot Hybridization:

Extraction of Total RNA:

Total RNA from healthy potato and PSTVd-infected leaves were extracted using Promega Kits (Corporation, Madison, USA). The UV spectra of RNA were min 230 nm and max. 260 nm and the ratio of $A_{260/280}$ was 1.0268. The RNA concentration was 23.0 µg/µl.

PSTVd cDNA Synthesis and Polymerase Chain Reaction:

DNA primers specific for PSTVd were constructed based on CCR of PSTVd nucleotide sequence *(Levy et al., 1994 and Shamloul et al., 1997).* The antisense primer (C) was complementary to 69-88 nt. (5`-CCCTGAAGCGCTCCTCCGAG -3`) and the sense primer (H) was homologous to 89-113 nt. (5`-ATCCCCGGGGAAACCTGG AGC GAAC-3`) (Amersham Pharmacia Biotech.). For first stranded cDNA synthesis, purified total RNA (1 µg) was mixed with 50 pmol of primer C, heated to 95°C for 3 min and quickly cooled on ice. Reverse transcription of PSTVd-RNA was carried out in a 20 µl reaction containing RNA the template and primer C, 500 µM each dNTPs, 200 unit of Molony Murine leukemia Virus (MMLV) reverse transcriptase (Promega). The mixture was incubated at 42°C for 45 min. PCR was carried out in a 50 µl reaction mixture containing 5 µl of cDNA reaction,10pmol each primer C and H, 200 µm of each DNTPs, 1.5 mM $MgCl_2$ and 2.5 unit of Taq DNA polymerase (Promega) in the reaction buffer provided by the same source. The mixture was incubated for 5 min. at 85°C for initial denaturation followed by thermal cycling for 40 cycles of 45 sec. at 94°C for denaturation, 45 sec at 62°C for annealing and 1 min. at 72°C for extension with final extension of 7 min. at 72°C followed by cooling at 4°C (Biometra).

Analysis of RT-PCR Amplified Product:

Amplified product (5 µl) were analysed by electrophoresis in a 1 % agarose gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at 100 volt for 1h. Gel was stained with ethedium bromide. DNA ladder marker (Promega) was used to determine the size of RT-PCR amplified products.

Preparation of cDNA PSTVd Probe:

The obtained RT-PCR product was labeled with non-radioactive digoxigenine (DIG) using the Genius TM System (Boehringer Mannheim Corp.) and its instructions. DNA fragment (359 bp) was amplified by PCR. The following components were added to a sterile microcentrifuge tube and placed on ice during pipetting: 5 µl 10 X PCR buffer without $MgCl_2$; 1.5 mM $MgCl_2$ stock solution; 5 µl 10 mM dNTP labeling mixture, 1 µl upstream and downstream primer (10 pmol) ;32.5 µl H_2O; 0.5 µl Taq DNA polymerase; 5 µl template cDNA. The volume was completed to 50 µl with dsH_2O. The reagents were vortxed and centrifuged and amplified in thermal cycler (Biometra Inc). Cycling parameters for the reaction were as follows: initial denatured at 95°C for 3 min before the first cycle. Denatured at 94°C for 45 sec., annealed at 62°C for 45 sec., extend at 72°C for 1 min. for 40 cycles with final extension at 72°C for 7 min.

Nucleic Acid Hybridization:

The capillary transfer of plant DNA from the gel to nylon membrane support was achieved using the southern technique *(Southern, 1975).* Nucleic acid hybridization was done according to the manufacturers instruction (Boehringer Mannheim GmbH, Mannheim, Germany).

Sequencing and Computer Analysis:

Nucleotide sequencing of the PCR amplified product of PSTVd-$_{EG}$ strain for the upper central conserved region which amplified with homologus and complementary primers the bands were excised; the purification and nucleotide sequencing was carried out at VACERA using ABI PRISM sequencer model 310, version 3.4. The sequence data, multiple alignments, phylogenetic relationship were analyzed by CLC RNA Workbench, Denmark.

Secondary Structure of PSTVd:

The Secondary structure of PSTVd was carried out using Vienna RNA package programme V.1.7, Institute for Theoretical Chemistry, University of Vienna (http://rna.tbi.univie.ac.at//cgi-bin/RNA).

RESULTS

The PSTVd-$_{EG}$ RNA template was reverse transcribed by MMLV reverse transcriptase. The resulting cDNA was amplified by PCR after adding primer specific for CCR. The PCR product was analysed on agarose gel electrophoresis. The expected size of amplified PSTVd-cDNA was, approximately~359 compared with positive control, whereas the total RNA of healthy potato tissue was not amplified by RT-PCR (Figure 1).

Southern-blot hybridization was used to confirm the authenticity of the PCR product of PSTVd obtained in (Figure 1). This was done by resolving the PCR product on agarose gel and transferred onto nitrocellulose membrane. Hybridization was performed by using PSTVd specific DIG-labeled c-DNA CCR probe. The DNA probe was successfully hybridized with all PCR products of full length of PSTVd amplified as shown in (Figure1). It was observed that major DNA fragment of the expected size ~ 359 bp amplified from PSTVd infected potato tissues.

Dot-blot hybridization was used to confirm PSTVd-isolate using DIG-labeled PSTVd cDNA to RT-PCR products amplified from total leaf RNA extracts of PSTVd infected potato plants, healthy and positive control. Positive hybridization was only obtained with PSTVd infected potato plants as positive control. But the healthy plants gave negative reaction (Figure 2).

The partial nucleotide sequence of the PSTVd-$_{EG}$ isolate was analyzed using primer specific for CCR (Figure 3) and recorded in GenBank under Accession number EU273604. The primary structure of RNA-PSTVd-$_{EG}$ strain is sequenced by the nucleic acid sequencer (Figure 3). While, the secondary structure of RNA-PSTVd strain was carried by Vienna RNA package programme V.1.7 (Figures 4, 5 and 6).

The minimum free energy of a secondary structure of RNA-PSTVd isolate is determined from its primary sequence by summing the energy contribution of all base pairs, interior loop, hairpin loop, multi-branched loop, bulge loop and external loop at 37°C using was -116.51 kcal / mol. It is Y-shaped structure composed of alternating single-and double-stranded regions (Figure 5). The bracket notation for RNA-PSTVd isolate secondary structure can be represented in the space- efficient bracket notation (Figure 4).The characters "(and)" corresponding to the ΄5 base and 3΄ base in the base- pair, respectively. While, "." denotes an unpaired base.

ΔG°_{37} (Gibbs free energy) of stalking pairs and interior, hairpin and bulge loops depend on predicated free-energy values (Kcal/mol at 37°C). It was observed that Base pairs lead to free energies < 0 while bulges and loops lead to free energies > 0.Consequently total free energy of PSTVd-isolate was -116.51 kcal / mol according to *(Serra et al., 1995)*.

Partition function (Q) of PSTVd-$_{EG}$ strain sum is all free energies of secondary structure. This can be calculating base pairing probabilities for each pair of bases (Figure 7).

Multiple alignment of PSTVd with five geographically distinct PSTVd isolates available from GenBank revealed high sequence identity (89-84%) using BLAST maintained at the NCBI NIH, Bethesda, MD. High level of sequence identity was 89% with isolate M25199 from Netherlands, 88% with strain M88681 from India and YO9576 from Poland 87% with isolate AF536193 from Australia and isolate EU257478 from Russian (Figure 8). Phylogenetic tree reveals high degree similarity to the other five isolates of PSTVd (Figure 9).

Statistical Analysis:

Statistical analysis of alignment sequence of PSTVd-$_{EG}$ with five geographically distant PSTVd- strains available from Genbank (Table 1) revealed that molecular characters PSTVd-EG strain, i.e. the molecular weight (98.077 kDa), base composition (A: 82, C:87, G:89 and T:59), frequencies nucleotides (A: 0.256, C: 0.272, G: 0.278 and T: 0.184) and % ratio A/T (1.3898) and C/G (0.9775) (Table 1).

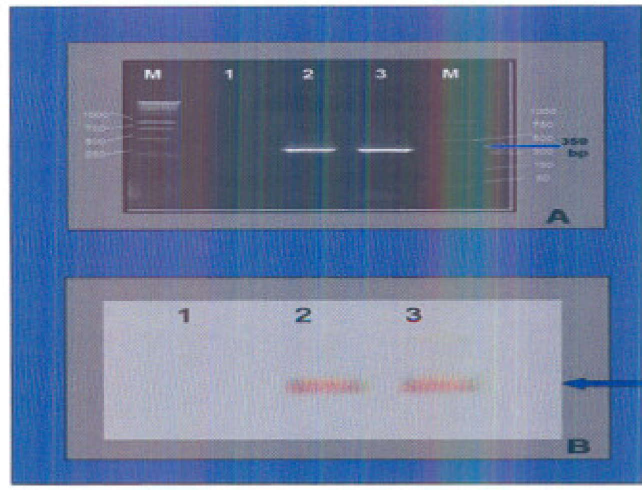

Figure 1: Amplified PSTVd cDNA product analysis (A) by 1% Agarose gel electrophoresis (B) Southern-blot hybridization, the expected band ~ 359 bp.

Lane M: DNA marker.Lane 1: Total RNA extracted from healthy potato leaves.

Lane 2: Positive control.

Lane 3: Total RNA extracted from infected potato leaves.

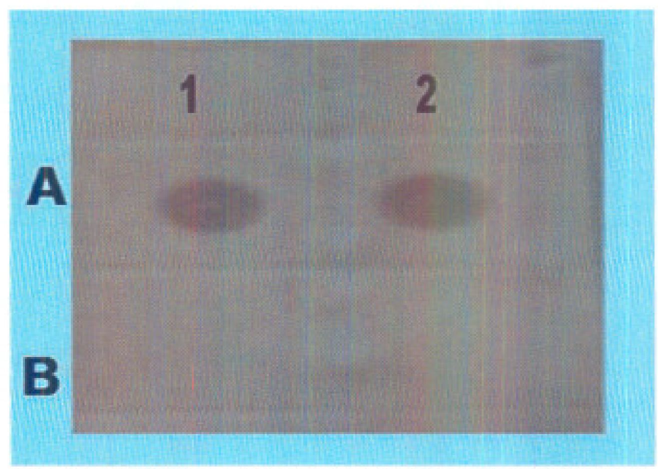

Figure 2: Dot-blot hybridization assay showing the colored spots of hybridized amplified PSTVd- cDNA product with DNA-probe labeled with DIG-11-dUTP. PSTVd-cDNA (Lane A_1), Positive control (Lane A_2), negative control (Lane B_1 and B_2).

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UCGNGAANAUCAGGUGUGAACCACAGGUACCACGAGUUUAGUUCCGAG
GAACCUCUGCGGUUCCAAGGGCUUAACUCCCUCCCCCCGAAGCCAAGAG
AUAGAGAAAAAGCCGGUCUCGGGAGCUUCACUUGGUUCCACCCGGUAA
UAACCCAAACGACCGCGCAAAGGGGGCGAGGGGUGGUCCUGCGGGCGC
GAGGAAGGACACCCGAACAAAGGAAGGGUGAAAACCCCGAUUCUUUUG
GAAUUACUCCUGUAGGCCGCUGGGCACUCCCCAAAAGACCUUAUUGCCA
GUUCGGUUCAGGUUUCCCCGUGGNAUAAAA
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Figure 3: Partial nucleotide sequence of primary structure of RNA-PSTVd-$_{EG}$ strain recorded under accession number of EU273604.

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Figure 4: Secondary structure of PSTVd-$_{EG}$ strain by dot brackt notation "(and)" corresponding to the ΄5 base and 3΄ base in the base- pair, respectively, "." denotes an unpaired base.

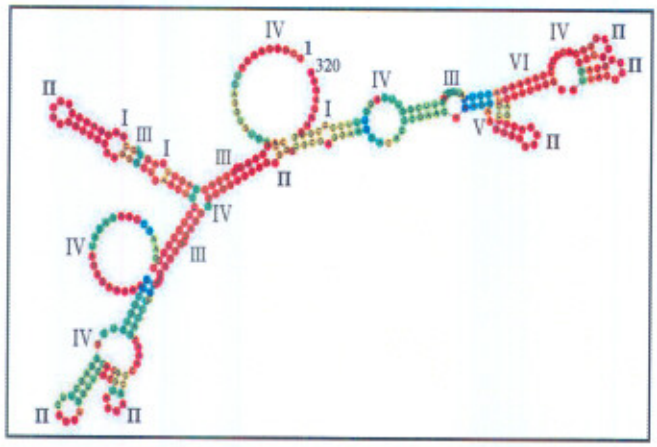

Figure 5: The Secondary structure of RNA PSTVd strain is Y-shaped structure. I=Interior loop, II=Hairpin loop, III=Bulge loop, IV= multi-branchedloop, V=External loop, VI=Stalk pairs

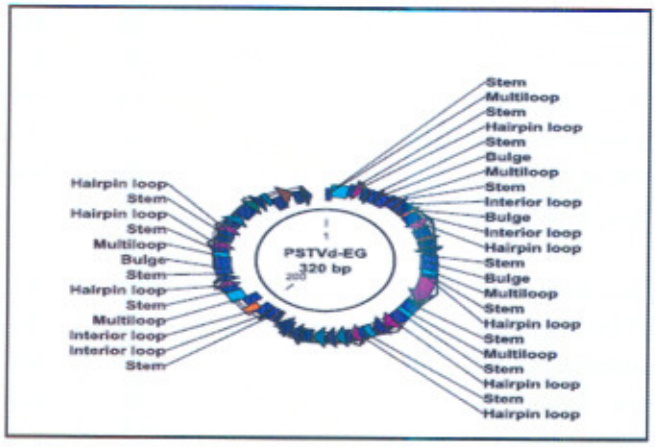

Figure 6: Diagrame illustrated distribution of secondary structures on circular RNA- PSTVd.

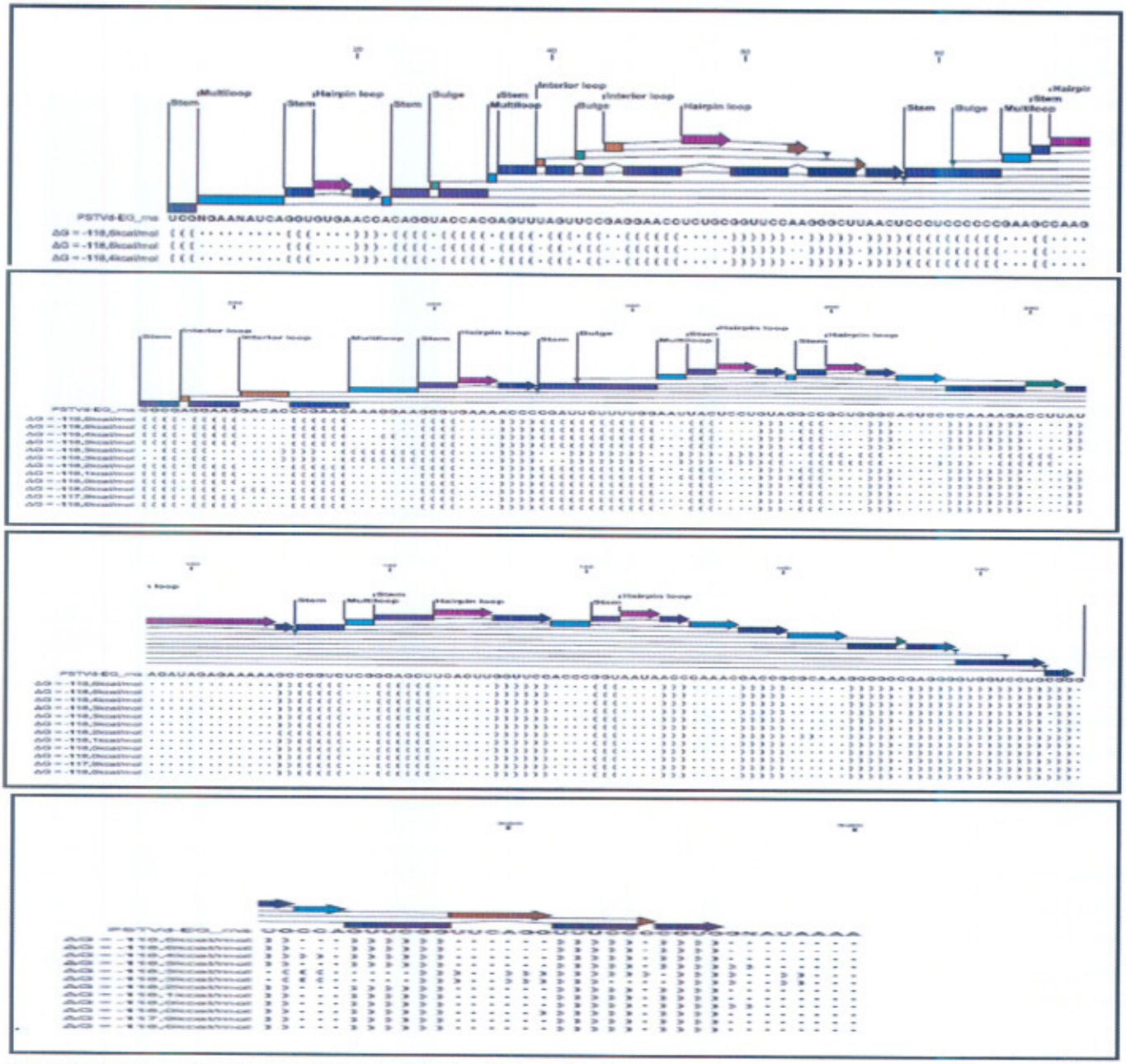

Figure 7: Create plot of marginal base pairing probabilities of all possible base pairs.
; unbase pair region.
; Base pair region.

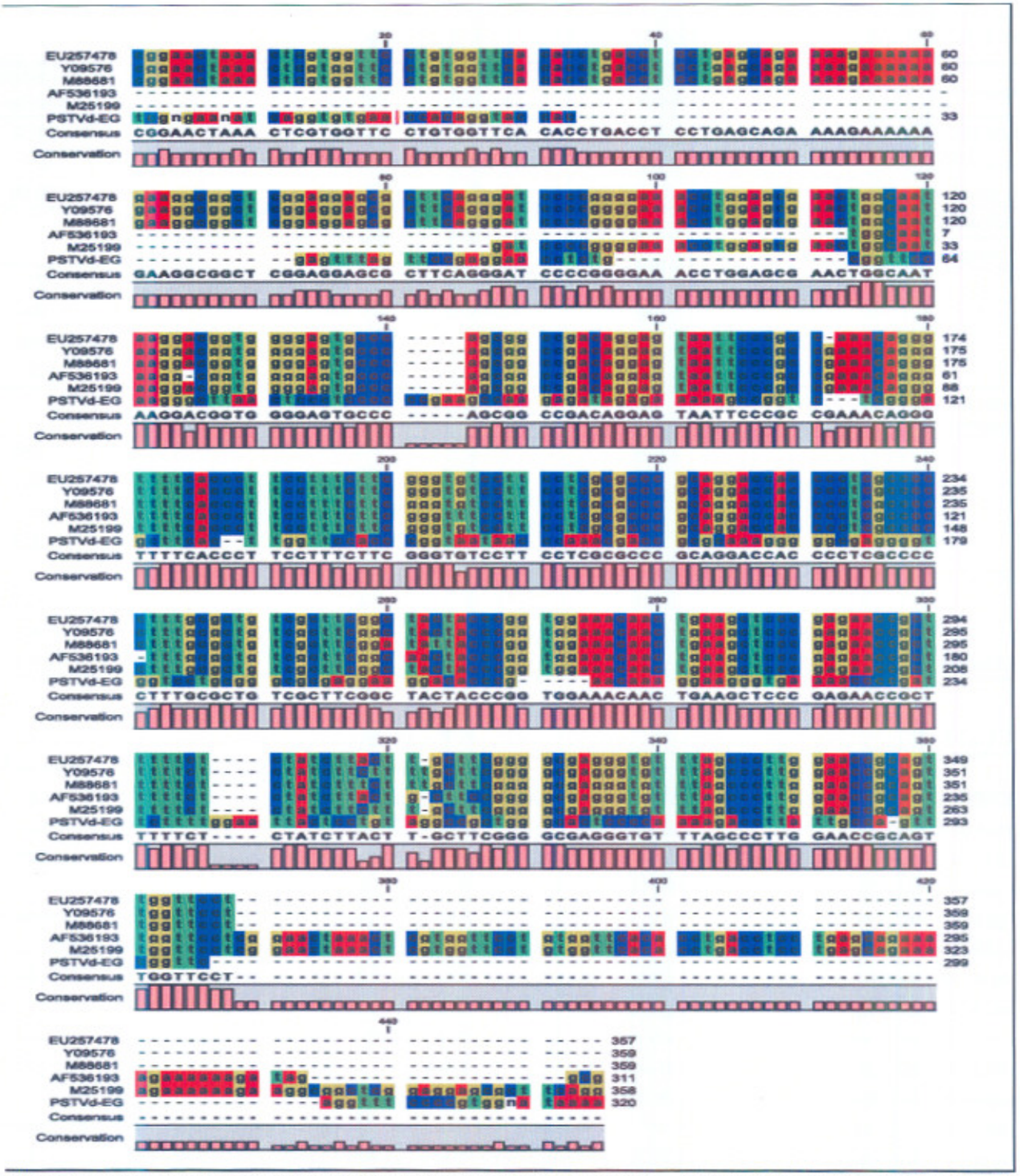

Figure 8: Multiple sequence alignment of the partial nucleotide sequence for the PSTVd-$_{EG}$ strain with the corresponding sequence of other PSTVd strains available in GenBank.

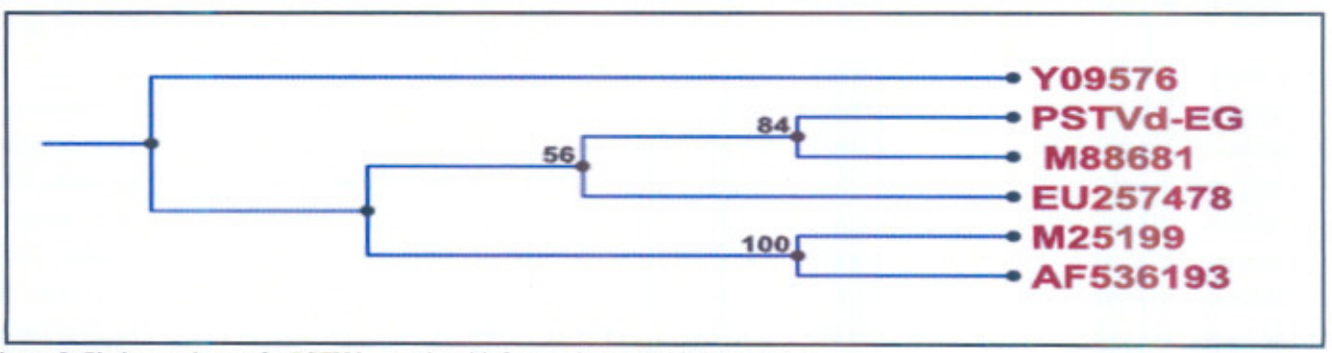

Figure 9: Phylogenetic tree for PSTVd$_{-EG}$ strain with five strains available in GenBank.

Table 1: Comparison between PSTVd-$_{EG}$ strain and strains available of GenBank in aligment sequence.

Comparison	PSTVd-$_{EG}$ (Egyptian)	AF536193 (Australia)	M25199 (Netherlands)	Yo9576 (Poland)	M88681 (India)	EU257478 (Russian)
Nucleic acid (N.A)	DNA	DNA	DNA	DNA	DNA	DNA
Lengt h of N.A (nt)	320	311	358	359	359	357
M.W (kDa)	98.077	94.629	109.191	109.479	109.503	108.887
No. of nucleotides						
base composition						
A	82	61	70	70	71	71
C	87	95	108	108	106	108
G	89	82	101	101	101	100
T	59	73	79	80	81	78
Frequencies nucleotides						
A	0.256	0.196	0.196	0.195	0.198	0.199
C	0.272	0.305	0.302	0.301	0.295	0.303
G	0.278	0.264	0.282	0.281	0.281	0.280
T	0.184	0.235	0.221	0.223	0.226	0.218
% ratio						
A/T	1.3898	0.8356	0.8861	0.8744	0.8765	0.9128
C/G	0.9775	1.1585	1.0693	1.0711	1.0495	1.0821
mfe (kcal/mol)	-116.51	-127.10	-168.20	-170.10	-170.10	-164.16

DISCUSSION

The RT-PCR was used to detect PSTVd-$_{EG}$ strain in total RNA extracted of infected potato plants and amplified cDNA. The expected size length of amplicon was approximately ~359 bp using primer design for CCR, in related to DNA ladder. These findings were confirmed by *Herold, et al. (1992), Wassengger et al. (1994) and Bostan, et al. (2004).* Primers of such wide specificity are usually targeted to CCR of the viroid genome that are likely to be highly conserved among members of a genus *Pospiviroid.* These primers have been selected to allow amplification of all isolates belonging to species and it could be potentially discrimination of viroid variants or strains within a viroid species *(Hadidi et al., 2003).* Southern-blot and dot-blot hybridizations were used to confirm the PCR product of PSTVd. The 359 bp amplified cDNA obtained from infected tissue hybridized with DIG-Labeled PSTVd-cDNA. No hybridization was observed from uninfected tissue.

These results were in agreement with *Daròs, et al. (1994)* who mentioned that southern (DNA gel blot) hybridization is an essential tool for basic research on viroids and it has been especially used to study the intermediate of the replication cycle of these pathogens. *Hadidi, et al. (2003)* mentioned that molecular hybridization has become important tool in detection of viroid since serological methods can not be applied to detect viroids. Molecular hybridization technique may be considered among the best options for detecting these pathogens and with PCR technology as the natural way to introduce molecular tools at the commercial level. PSTVd-cDNA probe of 350 nt bases was used to detect one picogram of the viroid by dot-blot hybridization technique in crude potato and tomato tissue extracts. Hybridization signal could be detected as little as 75 µg of infected plant tissue. The labeled PSTVd-RNA with digoxigenin to detect of PSTVd by dot-blot hybridization assay revealed that DIG-labeled the probe was highly sensitive specific and detecting as little as 2.5 Pg of PSTVd-RNA. As stated above viroids do not code for any protein so serological methods can not applied for their diagnosis *(Querci, 1990; Singh, 1991, Nakahara, et al. 1998 and Boer, et al. 2002).*

Thus, only biological, biochemical (Return and Sequential-PAGE) or molecular methods (molecular hybridization, RT-PCR etc.) can be used to detect viroid infections *(Hadidi et al., 2003).* The partial nucleotide sequence of PSTVd-$_{EG}$ isolate was analyzed using specific primer for CCR. The minimum free energy of a secondary structure for RNA-PSTVd isolate was -116.51 kcal/ mol which determined from its primary sequence by summing the energy contribution of all base pairs, interior loop, hairpin loop, multi-branched loop, bulge loop and external loop at 37°C using Vienna RNA package programme V.1.7. It is Y-shaped structure composed of alternating single-and double- stranded regions. *Serra, et al. (1995)* mentioned that RNA molecule folds into that structure with the minimum free energy (mfe). Free energy models typically assume that the total free energy of a given

secondary structure for a molecule is the sum of independent contributions of adjacent, or stacked, base pairs in stems (which tend to stabilize the structure) and of loops (which tend to destabilize the structure). Multiple alignment of PSTVd$_{EG}$ with five geographically distinct PSTVd isolates available from GenBank revealed high sequence identity (89-86%). High level of sequence identity was 89% with isolate from Netherlands, 88% with Indian, Polandian and Russian isolates. From previous sequence analysis found that molecular weight of PSTVd$_{EG}$ isolate was 98.077 kDa, base composition (A: 82, C: 87; G: 89 and T: 59), frequencies nucleotides (A: 0.256, C: 0.272, G: 0.278 and T: 0.184) and % ratio A/T (1.3898) and C/G (0.9775). Singh et al. *(1999)* 40 isolates of PSTVd from (potato, other Solanum species and greenhouse tomato) have sequence similarities in the range of 95-99%.

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