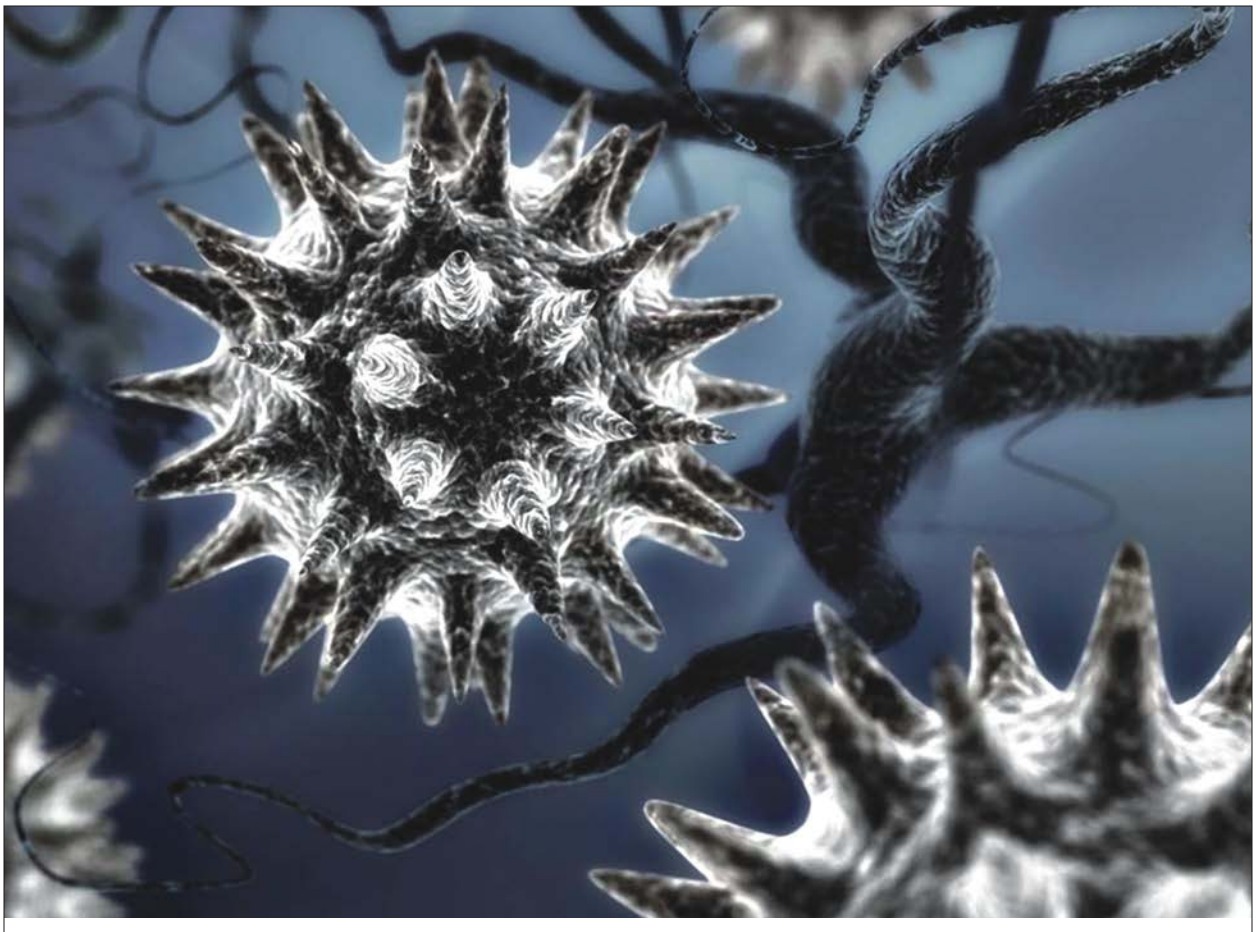

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Molecular Variation of Potato virus Y Isolated from Egypt

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ABSTRACT

Potato Virus Y (PVY) is one of the most important viruses affecting cultivated potatoes in Egypt. Different potato plants were collected from an experimental station in Giza Governorate, Egypt and were tested using RT-PCR. PVY was amplified using primers represented portion of the Coat Protein (CP) gene and 3' Untranslated Regions (UTR). Phylogenetic tree showed two main strain groups: Group I regroups PVY^{N} and PVY^{NTN} stains while Group II includes PVY^{O}, PVY^{W} and $PVY^{N:O}$ strains. The Egyptian PVY isolate was clearly classified within group I and was more closely related to PVY^{NTN} strains. Ten nucleotide substitutions resulted in 3 conserved amino acid substitutions ($V_1 \rightarrow I$, $G_7 \rightarrow E$, M or V and $S_8 \rightarrow G$) and were able to differentiate between both groups. The partial coat protein region was more diverse than that of the 3'UTR (92.6-100% and 97.7-100% identity, respectively). The 3'UTR of the Egyptian isolate showed RNA secondary structures different from those of the 5 PVY strains.

Key words: PVY, RT-PCR, phylogeny, CP, 3´UTR, strain groups

INTRODUCTION

PVY belongs to the *potyvirus* genus, Potato virus Y is an important pathogen in Solanaceous crops. The level of damage to crop is determined by the strain of PVY infecting the plants, the viral load, the time at which infection occurs as well as the tolerance the host possesses toward the virus (Warren *et al*., 2005). PVY is naturally transmitted by aphids in a non-persistent manner with great efficiency, causing epidemics in potato, tomato, pepper, tobacco and other solanaceous plants (De Bokx and Huttinga, 1981).

PVY has a single positive-sense genomic RNA~10 kb long and forms flexuous virions. The genomic RNA contains a unique ORF encoding a polyprotein which is processed into functional viral proteins by virus-encoded proteases (P1, HC-Pro and NIa (Riechmann *et al*., 1992)). The CP gene is the gene most frequently used for studies of genetic diversity in *Potyviruses* (Shukla *et al*., 1994).

Based on symptomatology in tobacco plants, PVY isolates were divided into two major pathology groups: the common or ordinary strain, PVY^{O} that induces mosaic or vein clearing symptoms in tobacco; and the necrotic strain, PVY^{N} that induces systemic Vein Necrosis (VN) in tobacco (De Bokx and Huttinga, 1981), Subsequently it has been recognized that each of these two strains contain subgroups and that other distinct strains do exist (Singh *et al*., 2008). In the necrotic, PVY^{N} group, two main recombinant types were identified initially: PVY^{NTN} and $PVY^{N\text{-}Wi}$ (Nie and Singh, 2003; Piche *et al*., 2004). PVY^{NTN} was first reported from Hungary in 1984 as a distinct subset of

isolates within the PVY^N strain that were capable of inducing tuber necrosis in potato tubers, often referred to as Potato Tuber Necrotic Ringspot Disease (PTNRD) (Beczner *et al*., 1984).

Several studies based on the molecular and phylogenetic characteristics of the PVY strains were previously performed by studying the different coding and non-coding regions of the genome (Boonham *et al*., 1999; Fanigliulo *et al*., 2004). The 3' Untranslated Regions (UTR) and Coat Protein (CP) coding sequences have been used for the identification and classification of many plant viruses (Yun *et al*., 2002). In this study, RT-PCR was used for the specific detection of PVY in potato samples. We analyzed and compared the nucleotide and the deduced amino acid sequence of C-terminal portion of the CP gene and entire 3' UTR of an Egyptian PVY isolate with those of previously reported. Phylogenic and secondary structure analysis was conducted to differentiate between PVY groups.

MATERIALS AND METHODS

Virus source: Eight potato samples (leaves and tubers) showing viral infection were collected from an experimental station in Giza Governorate. PVY isolate exist in our laboratory was used as a reference in RT-PCR.

RNA extraction: Total RNA extraction was done using extraction kit (RNeasy Mini Kit IAGEN# 74903).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): PVY was amplified using primers represented C-terminal portion of the CP gene and entire 3' UTR (Table 1). Reverse transcription and PCR were carried out according to the manufacturer's recommendations using ONE STEP RT-PCR kit (QIAGEN). The RT-PCR conditions were 30 min at 50°C, 2 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C and 10 at 72°C. The amplified product was resolved by electrophoresis in 1% agarose gel.

Sequence analysis: The amplified PCR product of sample n° 7 corresponds to portion of CP gene and 3' UTR was purified using QIAquick PCR purification kit (Qiagne Inc., Mississauga, ON, Canada). DNA sequencing was carried out with the Taq dye terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems 373A sequencer. DNA sequencing was carried out in one direction using the PCR product specific primer PVYv. The sequence was edited using Chromas Pro Version 1.34 software. The Egyptian isolate of PVY was compared with PVY sequences in the NCBI database with the program BLAST. Sequences were manipulated using BIOEDIT, Nucleotide and amino acid sequences were aligned using the multiple sequence alignment program CLUSTALW (Thompson *et al*., 1994) implemented in MEGA software (Kumar *et al*., 2001). The phylogenetic relationships of sequences were inferred and compared using the neighbor-joining algorithm (Saitou and Nei, 1987) included in MEGA software (Kumar *et al*., 2001). Bootstrapping of 1000 replicates was carried out (Felsenstein, 1985).

Table 1: Primers for RT-PCR-amplification

Primers' name	Polarity	Nucleotides sequence	Fragment size	Reference
PVYv	Sense	5-GAT GTT GCA GAA GCG TAT AT-3	640 bp	Yun *et al*. (2002)
	Anti-sense	5-GTC TCC TGA TTG AAG TTT AC-3		

RESULTS

Potato virus Y detection using

RT-PCR: PVY was detected in 4 out of 8 samples using RT-PCR (Table 2). PCR fragment of about 650 bp corresponding to the C-terminal region of CP and 3' UTR portion was produced for samples No. 1, 5, 7 and 8, however, no PCR product was detected with the negative control (healthy tobacco leaves) (Fig. 1a, b).

Comparative sequence analysis: One out of 4 PCR-positive samples (sample No. 7) was sequenced. The sequence was edited using Chromas Pro Version 1.34 software, resulted in 593 nucleotides. The Egyptian isolate was compared with PVY sequences in the NCBI database with the program BLAST. Nucleotide sequence obtained in this study is deposited into GenBank

Table 2: RT-PCR results of the PVY-field collected infected samples

No.	Potato samples	RT-PCR
1	Diamond-tubers	faint+
2	Diamond-tubers	-
3	Diamond-tubers	-
4	Spunta-tubers	-
5	Spunta-tubers	+
6	Diamond- tubers	-
7	Diamond-tubers	+
8	Spunta-leaves	+

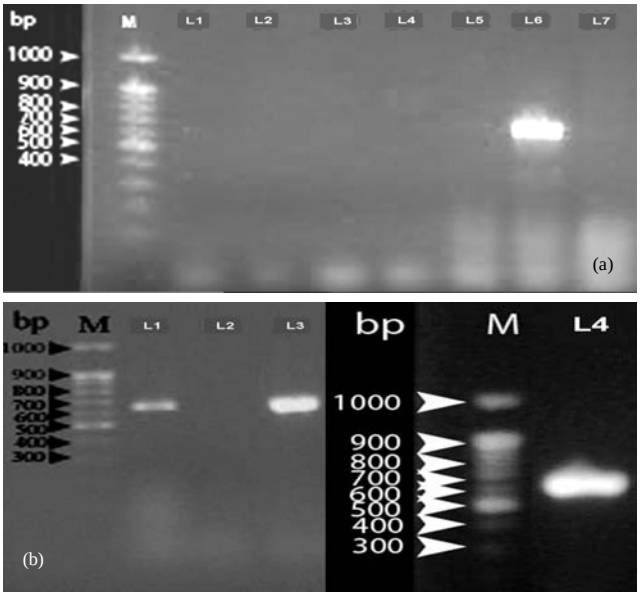

Fig. 1 (a-b): Agarose gel electrophoresis analysis of RT-PCR products of PVY-collected samples from field. (a) M: 100 bp DNA ladder. L1, 2, 3, 5 and 6 correspond to samples 1-5, respectively. L4 and 7: Negative control (healthy tobacco leaves). (b) L1: Positive control (PVY-infected tobacco leaves collected from our greenhouse); L2-L4 correspond to samples 6, 7 and 8, respectively

(accession number JF698682). The isolate named "PVY-Egypt-Medhat". Thirty six sequences correspond to the 5 PVY strains: PVY^{N}, PVY^{NTN}, PVY^{O}, PVY^{W} and $PVY^{N:O}$ were retrieved from the GenBank database (accession numbers are shown in Fig. 2-4. Three more sequences (only coat protein sequence) correspond to 3 Egyptian isolates were retrieved from GenBank (AF522296.1, GU550076.2 and GU980964.1) were used in the comparison (El-Mohsen *et al*., 2003).

Ten nucleotide substitutions located at positions 1-39 at the CP portion were able to differentiate between the necrotic (PVY^{N} and PVY^{NTN}) and the ordinary ($PVY^{N:O}$, PVY^{O} and PVY^{W}) groups (Fig. 2-4). Our Egyptian isolate was located within the necrotic group, however, the 3 other Egyptian isolates retrieved from GenBank were classified within the ordinary group (Fig. 3, 4). Only 3 out of the 10 positions resulted in amino acid substitutions: (V_1→I), (G_7→E, M, or V) and

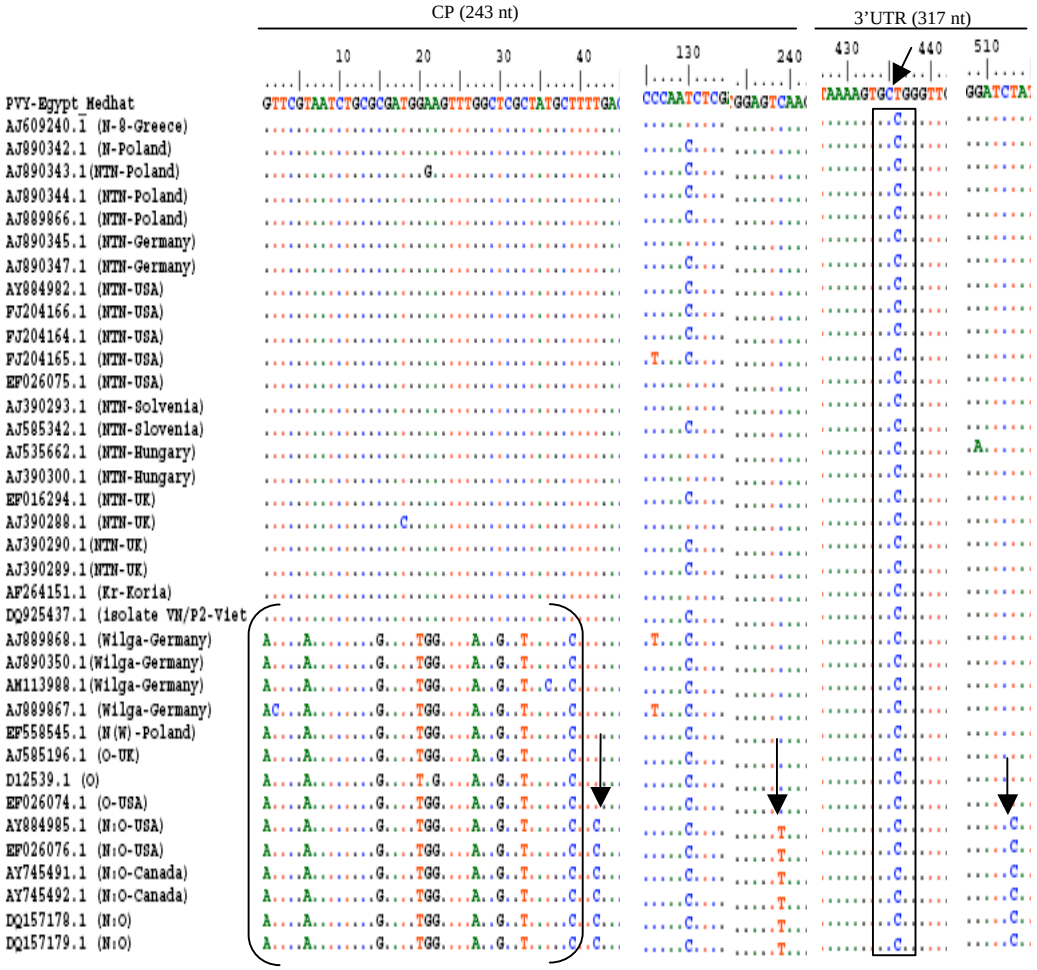

Fig. 2: Multiple alignments of the nucleotide sequence of partial CP region and the 3'UTR region of PVY isolates. Sequences related to group II, in comparison with group I, are parenthesized, however, the 3 positions characterize $PVY^{N:O}$ subgroup are arrowed. The single nucleotide substitution (T_{436}→C) located in the 3'UTR region and differentiate between the Egyptian and the other PVY isolates is boxed in bold. Numbers on top represent the partial CP and the 3'UTR region position. Only the differences are shown

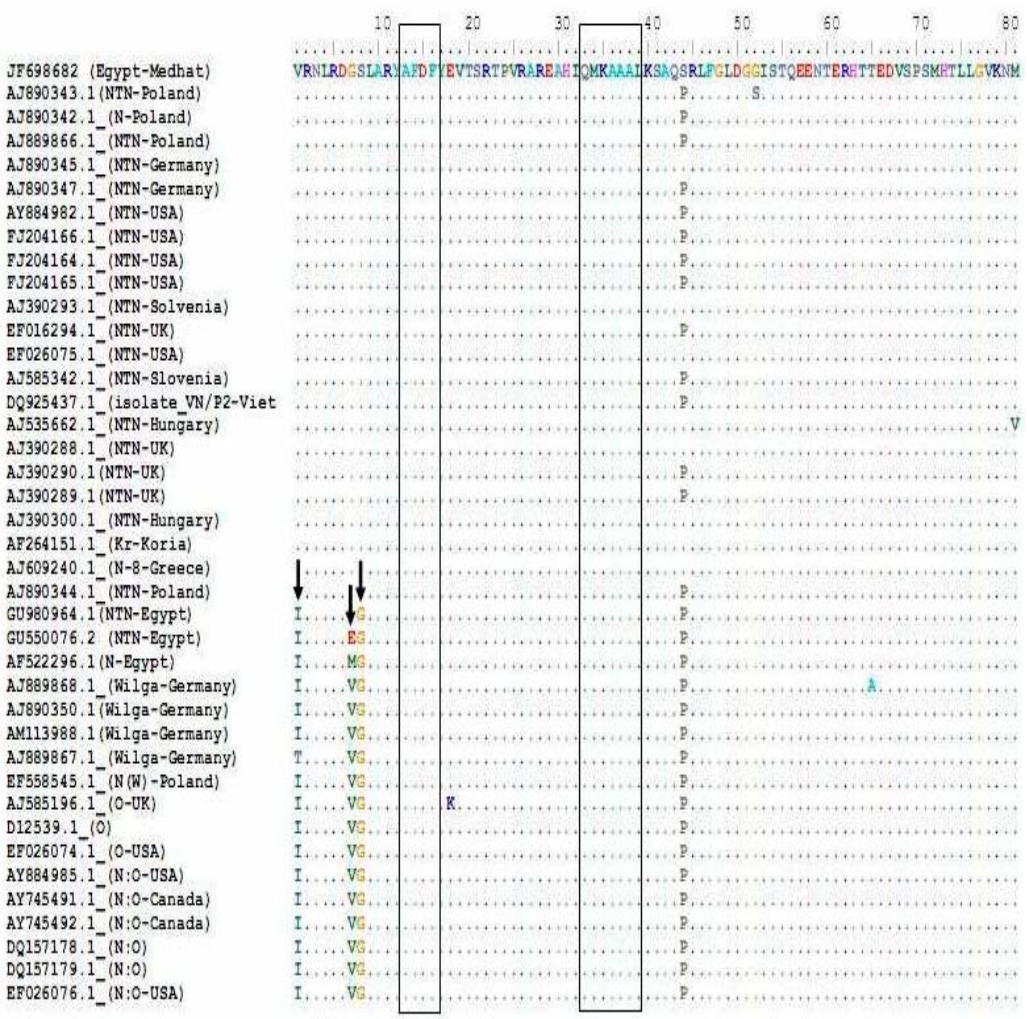

Fig. 3: Multiple alignments of the C-terminal region of the CP amino acid of PVY isolates. The consensus AFDF and QMKAAAL sequence motifs are boxed. Sequences related to group II, in comparison with group I, are arrowed. Numbers on top represent the deduced CP amino acid position. Only the differences are shown

(S_8→G) (Fig. 3). The CP region was more diverse than the 3'UTR nucleotide sequences (92.6-100% and (97.7-100% identity, respectively). However, the identity within groups was less diverse (96.3-97.5% and 97.7- 98.7% for CP and 3'UTR, respectively). $PVY^{N:O}$ subgroup was characterized by the presence of 2 nucleotide substitutions did not result in amino acid changes in the CP portion (T_{42}→C and C_{234}→T) and one nucleotide change in the 3'UTR region (T_{513}→C). Sequence motifs AFDF and QMKAAAL were found within the CP region (position 13 and 33, respectively) (Fig. 3). The Egyptian isolate of PVY was characterized by the presence of nucleotide substitution (C_{436}→T) located in the 3'UTR region and differentiate between our isolate and the other PVY isolates (Fig. 2). Interestingly, a substitution from S_{44}→P was detected in 14 out of 23 sequences of group I, however, this substitution was conserved in all sequences of group II (Fig. 3).

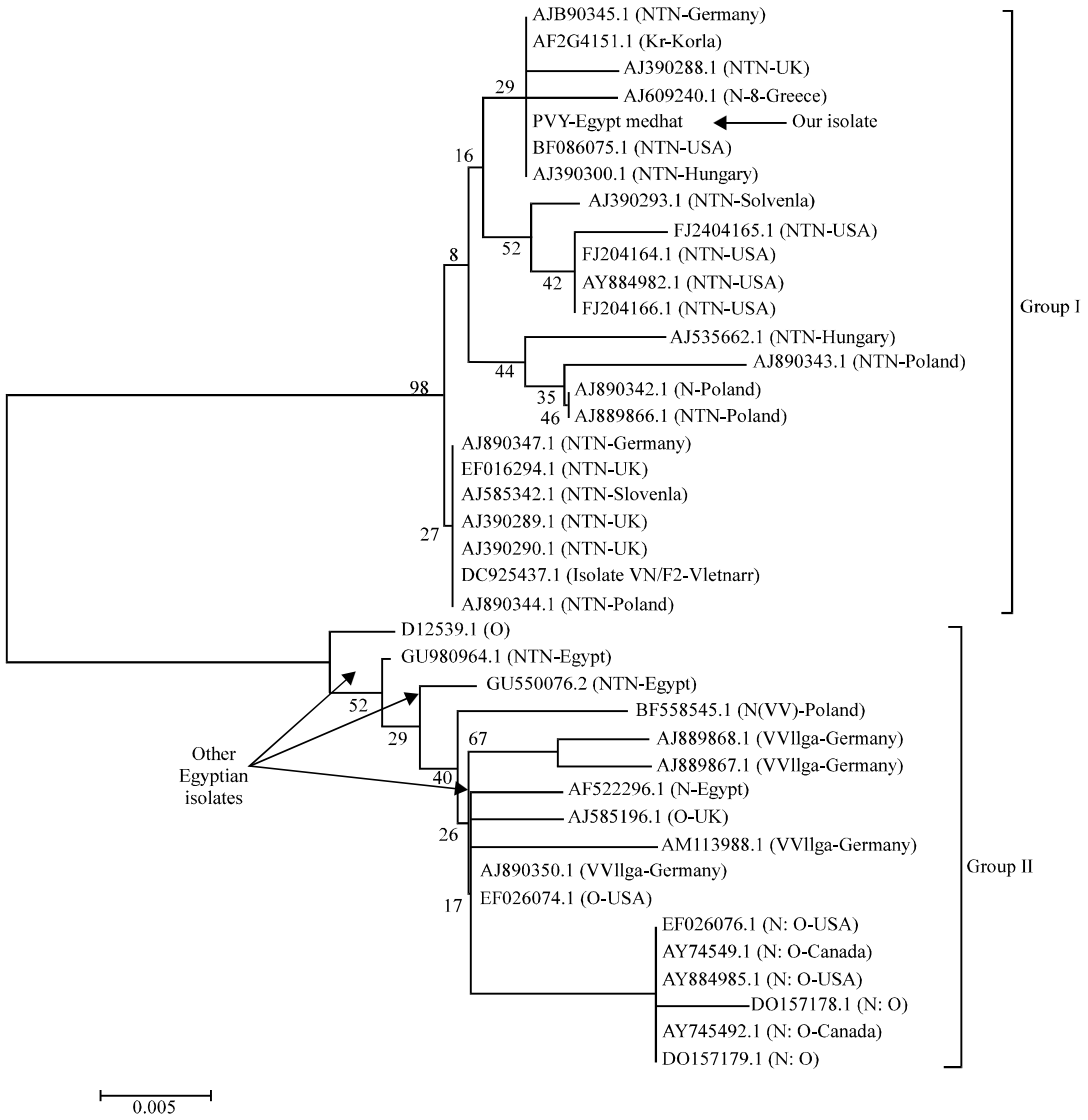

Fig. 4: Phylogenetic tree of the PVY Egyptian isolate and selected PVY isolates based on the analysis of the nucleotide sequence of partial CP region, The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

Phylogenetic analysis: We investigated in more details the genetic relationships of PVY isolates. The phylogenetic relationships of sequences were inferred and compared using the neighbor-joining algorithm (Saitou and Nei, 1987) included in MEGA software (Kumar *et al*., 2001). Bootstrapping of 1000 replicates was carried out (Felsenstein, 1985). The obtained phylogenetic tree using the nucleotide sequences of the CP divided the sequences into two main groups: Group I regroups PVY^{N} and PVY^{NTN} strains while Group II include $PVY^{N:O}$, PVY^{O} and PVY^{W} strains (Fig. 4). The phylogenetic profile was similar to the nucleotide and the predicted amino acid substitutions described above (Fig. 2, 3). The Egyptian isolate was clustered with group I and was more closely

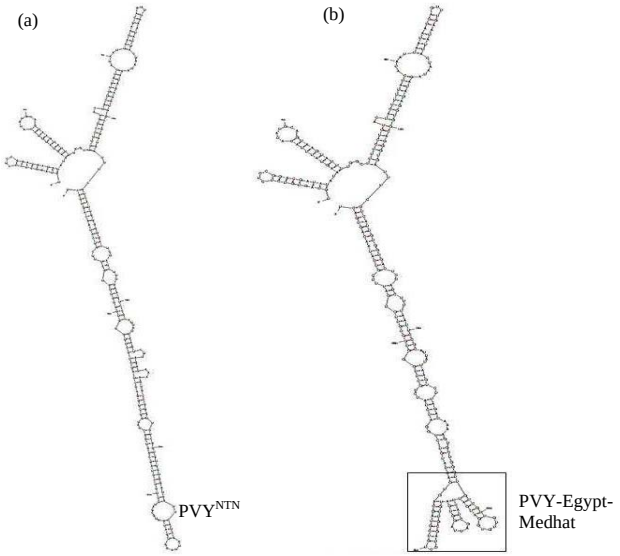

Fig. 5 (a-b): Comparison of the RNA secondary structures of the 3' UTR of the Egyptian PVY isolate and the consensus sequence of PVY^{NTN} subgroup, predicted by the use of the mFOLD version 3.2 program (Zucker, 1989). The RNA secondary structures of the Egyptian isolate differed from that of PVY^{NTN} consensus sequence by the presence of a multi-loop with 3 hairpins (boxed) instead of forming interior loop. RNA secondary structures obtained with the other strains was similar to that obtained with PVY^{NTN}

related to isolates: NTN-UK, NTN-Germany NTN-USA, NTN-Hungery, K- Koria and N-Greece (99.6-100% identity). However, the 3 other Egyptian isolates retrieved from GenBank were clustered with group II.

Secondary structure prediction: To investigate the possible changes in the predicted secondary structure of the consensus sequence of the 5 PVY strains in comparison with the Egyptian isolate, mFOLD version 3.2 program (Zucker, 1989) (http://mfold.bioinfo.rpi.edu/) was used. The RNA secondary structures of the Egyptian isolate differed from that of PVY^{NTN} consensus sequence by the presence of a multi-loop with 3 hairpins (boxed) instead of forming interior loop (Fig. 5). RNA secondary structures obtained with the other strains was similar to that obtained with PVY^{NTN}. The Egyptian isolate was characterized by the presence of nucleotide substitution (C_{436}→T) located in the 3'UTR region which differentiated between our isolate and the other PVY isolates. This substitution might be the reason of changes resulted in the secondary structures. No clear secondary structure changes were found using CP or 3'UTR between the 5 strains.

DISCUSSION

As expected in previously studies, PVY sequences were clustered in 2 groups (necrotic and ordinary groups) according to sequence alignment and phylogenetic analysis. These 2 groups did not correlate to the geographical distribution. They don't possess a spatial identity and can evolve and adapt from country to another, abstraction to the environmental conditions (Feki and Bouslama, 2008).

Specific differences were observed between the necrotic (group I) and the ordinary (group II) groups at the first 10 amino acids of the CP. No notable difference was observed between the PVY^{N} and the PVY^{NTN} strains. Group II was characterized by the presence of amino acid substitution S_{44}→P. Also, 3 nucleotide substitutions at positions 42, 234 and 513 distinguished $PVY^{N:O}$ subgroup. This differences my play a role in necrotic symptoms for group I and veinal necrosis in group II. This data was in agreement with those reported by Feki and Bouslama (2008). Interaction between the PVY CP and the plant proteins expression might play probably an important role in the virus symptoms (Feki *et al*., 2005; Hofius *et al*., 2007). It was described for numerous plant viruses, any sequences of the viral genome corresponding to open reading frames, regulatory elements, non-coding sequences or silent mutations could be involved in virus symptom induction (Van Der Vossen *et al*., 1996; Cecchini *et al*., 1997; Hirata *et al*., 2003). Contrarily, Yun *et al*. (2002) suggested that the C- terminal portion of CP and 3' UTR sequences does not always explain symptom variations; and symptom determinants for necrosis in tobacco leaves and potato tubers are located in the HC-Pro gene and in the NIa, NIb and N-terminal portion of CP, respectively.

In the 3' UTR of the Egyptian isolate, there was a substitution from cytosine to thymine at position 436. This substitution might be the reason of secondary structure change from interior loop exists in the 5 subgroups to a multi-loop with 3 hairpins in our isolate. Moury (2010) reported that 3 Chilean isolates were predicted to possess two stem-loop structures instead of one in groups N, O or C.

In this study, we showed that our Egyptian PVY isolate is clearly classified within group I, however, the 3 other previously reported Egyptian isolates were located within group II. The Egyptian isolate is more closely related to subgroup PVY^{NTN}. More biological studies will support this classification. Also, one nucleotide change in the RNA secondary structures characterized the Egyptian isolate; however, we need to study the effect of this substitution. It will be necessary to analyze genes or sequences involved in host selection and symptom developments.

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