Full-length sequence of Egyptian potato leafroll virus (PLRV) isolate

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ABSTRACT

Potato leafroll virus (PLRV) was isolated from Egyptian fields of potato. Overlapped reverse transcribed PCR products were amplified on the basis of polysomal RNA extracted from PLRV-infected Physalis floridana plants. These products were cloned and sequenced. Sequence reactions indicated that the Egyptian isolate of PLRV contains 5883 nucleotides. The sequence destination analysis between PLRV isolated from Egypt and the isolates of Scotland, Netherlands, Canada and Australia indicated 98.1, 97.9, 97.5 and 93.5% similarity, respectively. The comparison between the open reading frames of the Egyptian isolate and the Scottish isolate of PLRV indicated that ORF1 showed the highest degree (98.7%) of similarity, while ORF7 showed the lowest degree (97.1%) of similarity when compared to the corresponding frames. ORF0, ORF2, ORF3, ORF4, ORF5 and ORF6 showed 98.3%, 98.5%, 97.8%, 98.3%, 97.8% and 97.4% similarity, respectively, when compared to their responding frames of PLRV isolated from Scotland.

Key Words: *PLRV, full length sequence RT-PCR.*

INTRODUCTION

Potato leafroll virus (PLRV), a type member of poleroviruses (D'Arcy and Mayo, 1997), infects potatoes worldwide. Due to infection, potato plants show yellowing and rolling symptoms on the foliage part of the plants. Infected tubers show necrotic legions in the phloem tissue. The viral host range is restricted to members of family *Solanacae*. PLRV is transmitted via green peach aphids (*Myzus persicae*) in a circulative manner without any evidence for propagation in its invertebrate vector (Eskandari *et al.*, 1979). It is restricted to the phloem tissue of the infected plants (Harrison, 1984). PLRV particles are isometric with a diameter of 22-25 nm. It contains 30% of single stranded RNA with a molecular weight of ~5.9 kb and has positive sense properties (Mayo *et al.*, 1989). The viral genome is covalently linked to a 7.2 kDa protein (VPg) at its 5' start and not polyadenelated at the 3'end (Mayo *et al.*, 1982). The RNA codes for at least 8 open reading frames (ORFs) that are located in two clusters of genes and separated by 197 nucleotides (nt) of noncoding sequences. The first cluster is preceded by 174 nt of noncoding sequences, while the second cluster is followed by 141 nt of noncoding sequences (Mayo *et al.*, 1989). The first gene cluster is translated directly from genomic RNA to produce ORF0 with Mr of 28 kDa and responsible for the symptom development

(Van der Wilk *et al.*, 1997a), ORF1 with Mr of 70 kDa with a chymotrypsin-like serine protease domain (Gorbalenya *et al.*, 1989) and the VPg mapped to be downstream of the protease domain (Van der Wilk *et al.*, 1997b). ORF0 and ORF1 are overlapped in different frames. ORF2 with Mr of 118 kDa is produced as a frame-shift from ORF1 (Prüfer *et al.*, 1992) and contains RNA dependent RNA polymerase (RdRp) motifs. The second cluster is translated from subgenomic RNA1 (sgRNA1) and sgRNA2 with Mr of 2.3 kb and ~0.8 kb, respectively (Tacke *et al.*, 1990; Miller and Mayo, 1991; Ashoub *et al.*, 1998). SgRNA1 serves as a messenger RNA (mRNA) for the coat protein (ORF3) with Mr of 23 kDa and completely overlaps the17 kDa movement protein (ORF4) but in a different frame and the aphid transmission factor (ORF5) that is located directly downstream of the ORF3 amber stop codon and encodes a 53-kDa polypeptide (Tacke *et al.*, 1990; Miller and Mayo, 1991). SgRNA2 serves as mRNA for at least two ORFs. ORF6 present within the C-terminus of ORF5 but in other frame with Mr of 7.1 kDa and ORF7 representing the C-terminus of ORF5 with Mr of 14.1 kDa with a proposed transcriptional regulation function (Ashoub *et al.*, 1998).

Different isolates of PLRV were already sequenced. These include a Scottish isolate of PLRV (Mayo *et al.*, 1989), a Netherlands isolate (Van der Wilk *et al.*, 1989) and an Australian and a Canadian isolates (Keese *et al.*, 1990). In this report, the full-length sequence of PLRV isolated from Egypt is identified and characterized. Its relation to the published sequences of PLRV's is also investigated.

MATERIALS AND METHODS

Samples of potato plants grown under field conditions were collected from Kafr-El-Zayadt at CIP station, Egypt. Plants were tested for presence of PLRV using enzyme linked immunoabsorbent assay (ELISA) kit (Beohringer Mannheim) according to the manufacturer recommendations. Single positively infected plant was used as source for viral infection. *Myzus persicae* were fed on infected plant materials for 5 days acquisition period prior for transmission to *Physalis floridana* plants. A population of 5 aphids was used to transmit the virus into each *Physalis floridana* plant. After additional 5 days, plants were sprayed with insecticide to kill the aphids.

RNA Extraction

Polysomal RNA was extracted from *Physalis floridana* plants according to the method described by Leiser *et al.* (1992) as follows: Leaf material of PLRV-infected *Physalis floridana* plants was ground in mortar with pestle to fine powder under liquid nitrogen. Fine powdered plants were transferred to centrifuge tubes and 2 volumes of extraction buffer (200 mM Tris-HCl pH 9.0, 400 mM KCl, 35mM $MgCl_2$, 200 mM sucrose and 12.5 mM EGTA) were added. Extract was clarified with two cycles of extraction using an equal volume of phenol/chloroform. After centrifugation at 10,000 rpm for 10 minutes, the nucleic acid-containing-aqueous phase was collected. Nucleic acids were precipitated using 2 volumes of absolute ethanol. After centrifugation at 10,000 rpm for 15 minutes at 4°C, the pellet was resuspended in ¾ volume of 3M of sodium acetate, pH 5.2 and kept on ice for 30 minutes. RNA was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C. RNA pellet was resuspended in 1/10 volume H_2O and precipitated using 2 volume of absolute ethanol. After centrifugation at 10,000 rpm for 15 minutes at 4°C, pellet was washed with 100 µl 70% ethanol. RNA pellet was air dried and resuspended in H_2O.

Concentration of RNA was estimated at 260 nm and adjusted to a final concentration of 1 g/1 l.

cDNA and PCR reactions

Aliquots of 10 g polysomal RNA was reverse transcribed in the presence of 100-pmol of reverse primer. Table (1) shows primers sequences and their positions that are on the basis of Netherlands isolate of PLRV (Van der Wilk *et al.*, 1989). 10 mM dNTPs, 1X reverse transcriptase buffer and 200U of MMLV-reverse transcriptase (Promega) were added to each reaction in a final volume of 25 l. Reactions were incubated at 42 C for 90 minutes. After incubation, aliquots of 5 l cDNA products were used to generate the PCR products in the presence of 10 pmol forward primers, 10 pmol reverse primers (Table 1), 1mM $MgCl_2$, 10 mM dNTPs, 1X PCR buffer and 2.5U *Taq*DNA polymerase (BRL) in final volume of 50 l reactions. After DNA amplification, PCR reactions were purified using Quiax II gel extraction kit (Quiagen) according to the manufacturer recommendations. PCR products were incubated with 1 U of Klenow enzyme (Roch), 10 mM dNTPs and 1X Klenow buffer for 15 minutes at 37 C to blunt ending the PCR product. DNAs were incubated for 1 hr at 37 C with T4-polynucleotide kinase (Roche) in presence of 1X T4-PNK buffer and 10 mM ATP (Ausubel *et al.*, 1994). After purification as above, DNAs were cloned into the *Sma*I site of pSP72 (Promega). After transformation to *E.coli JM109* (Hanahan, 1985), colonies were screened for the targeted DNA using the alkaline lyses method (Ausubel *et al.*, 1994). Plasmids from positive clones were prepared using alkaline lysis method followed by PEG precipitation (Ausubel *et al.*, 1994) and sequenced using universal primers from the vector (Promega). Sequence reactions were carried out using 310 Sequencer (Applied Bio Systems).

The 5' transcriptional start of PLRV genome was determined using 5'-3' rapid amplification of cDNA ends (RACE) kit (Roche) according to the standard protocol described by the manufacturer using primer P4 for the cDNA reaction and primer P2 (Table 1) combined with the oligo-dt-Anchor primer (Roche) for the PCR reaction. After PCR purification, DNA was directly applied to the sequencing reaction using P2 primer for the reaction.

PLRV sequence was analyzed and compared to isolates from Netherlands, Scotland, Canada and Australia using Dnastar program (Expert sequence analysis software).

RESULTS AND DISCUSSION

An isolate of PLRV obtained from the Egyptian fields of potato was biologically purified and maintained on *Physalis floridana* plants as a source for viral infected materials. Overlapped reverse transcribed PCR products were amplified on the basis of polysomal RNA extracted from PLRV-infected *Physalis floridana* plants (Fig. 1,C & D). These products were cloned and sequenced. The 5' start sequence of PLRV was determined by a 3'-5' RACE reaction. The overlapping of PCR products is to exclude any sequences introduced by forward and reverse primers to PCR products used to generate the clones for sequencing reactions of PLRV genome.

Sequence reactions indicated that the Egyptian isolate of PLRV contains 5883 nucleotides (Fig. 2). The 5' transcriptional start of PLRV proved to be an adenosine residue. Comparison of the PLRV-5' first 18 nucleotides indicated an identical sequence for PLRV-Egypt, PLRV-Netherlands, PLRV-Australia and PLRV-Canada isolates. On the other hand, PLRV isolated from Scotland has a 104-nucleotide longer leader sequence. The

sequence destination analysis between PLRV isolated from Egypt and the Isolates of Scotland, Netherlands, Canada and Australia (Fig. 3) showed that Scottish isolate of PLRV has the highest degree of similarity (98.1%) followed by the Netherlands isolate (97.9%), Canadian isolate (97.5%), while the Australian isolate showed only 93.5% similarity when compared to the Egyptian isolate.

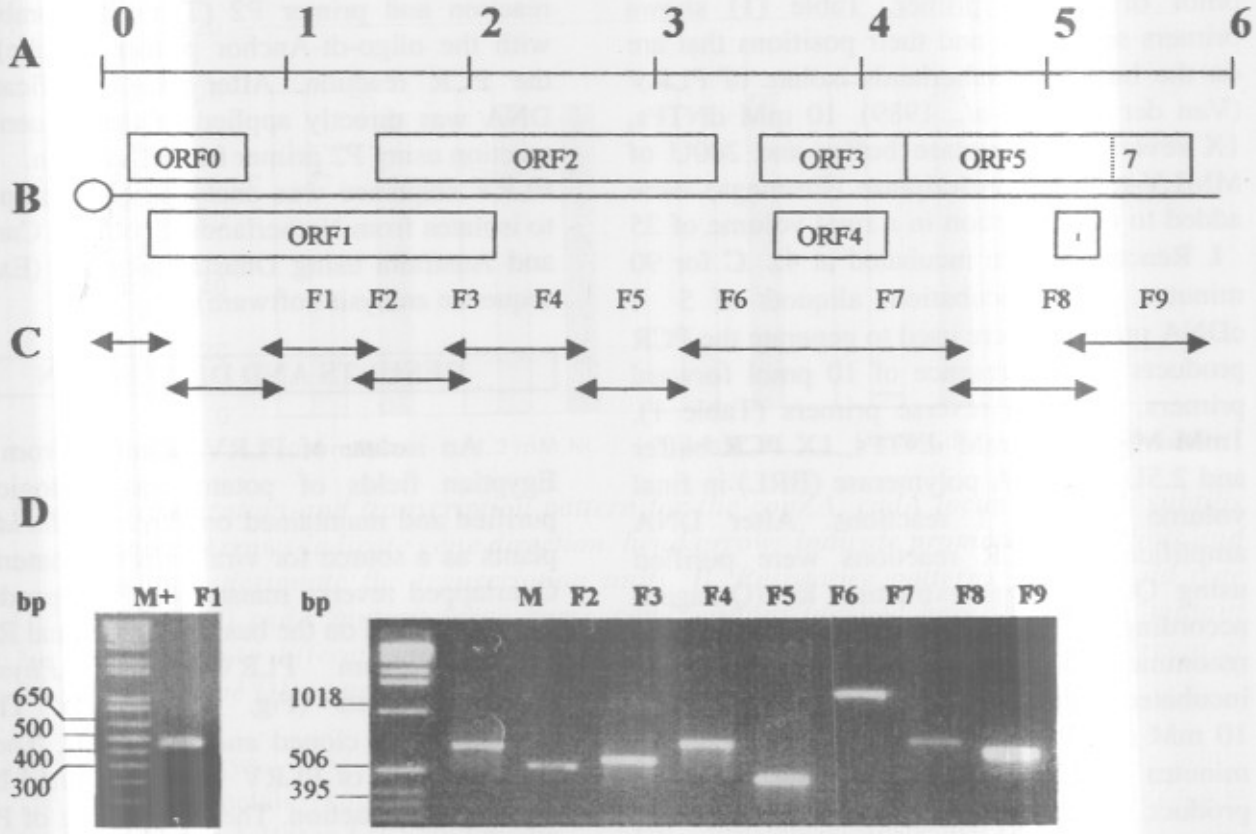


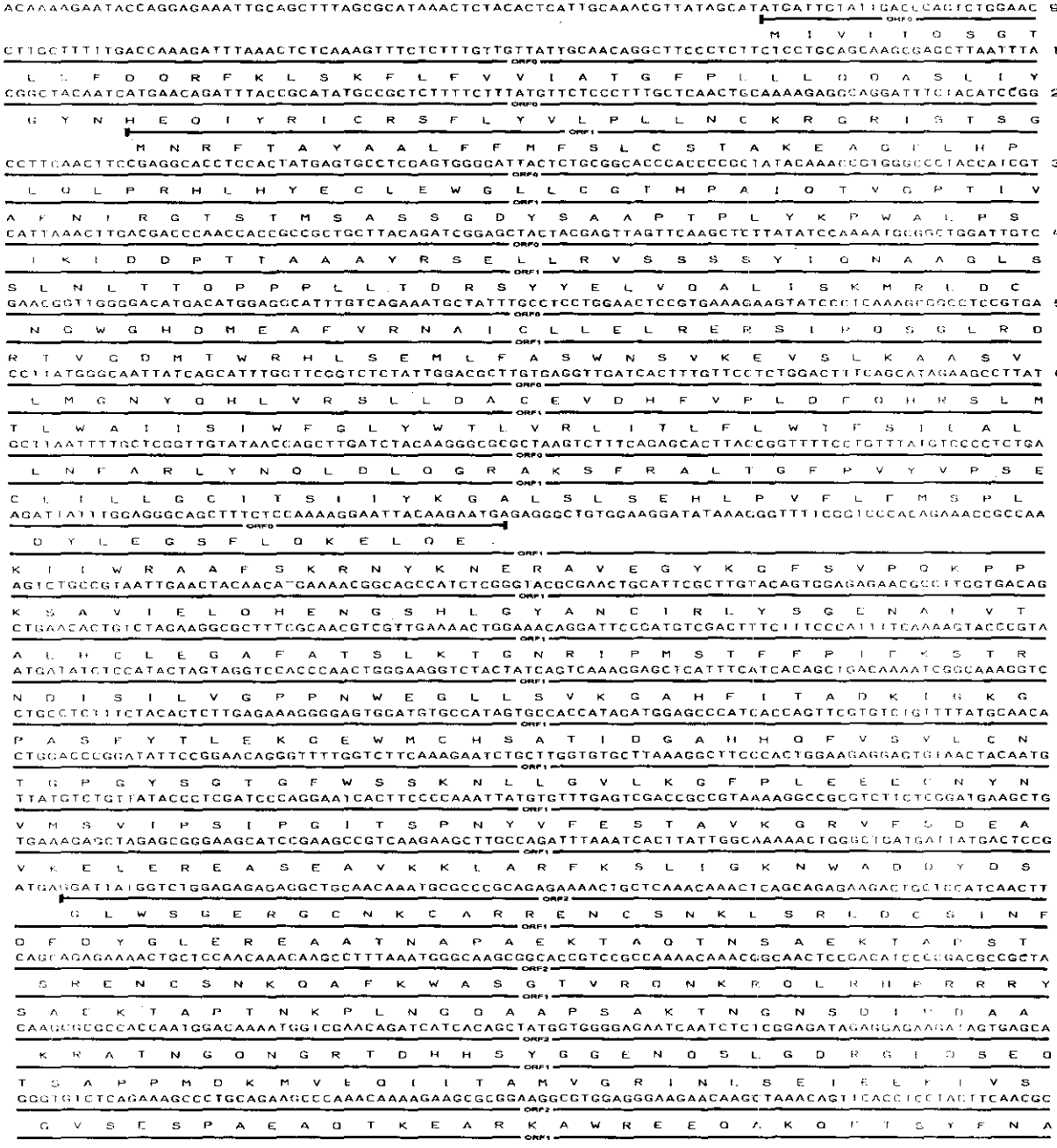
Fig. (1): (A) Molecular weight ruler to indicate the approximate size of the open reading frames (ORFs) represented by PLRV genome,as boxes, the circle indicates the VpG at the 5'start of PLRV genome (B). Schematic representation PCR products generated to cover PLRV genome (C). An autogram of 1.5% agarose gel stained with ethedium bromide shows the PCR products generated to cover the genome of PLRV (D).One Kb plus MW marker (M+,BRL), PCR product of the 5'start (F1) generated by the 3'-5' RACE Kit (Roch), PCR products covering PLRV genome excluding the 5'start (F2-F9) and One Kb MW marker (M, BRL).

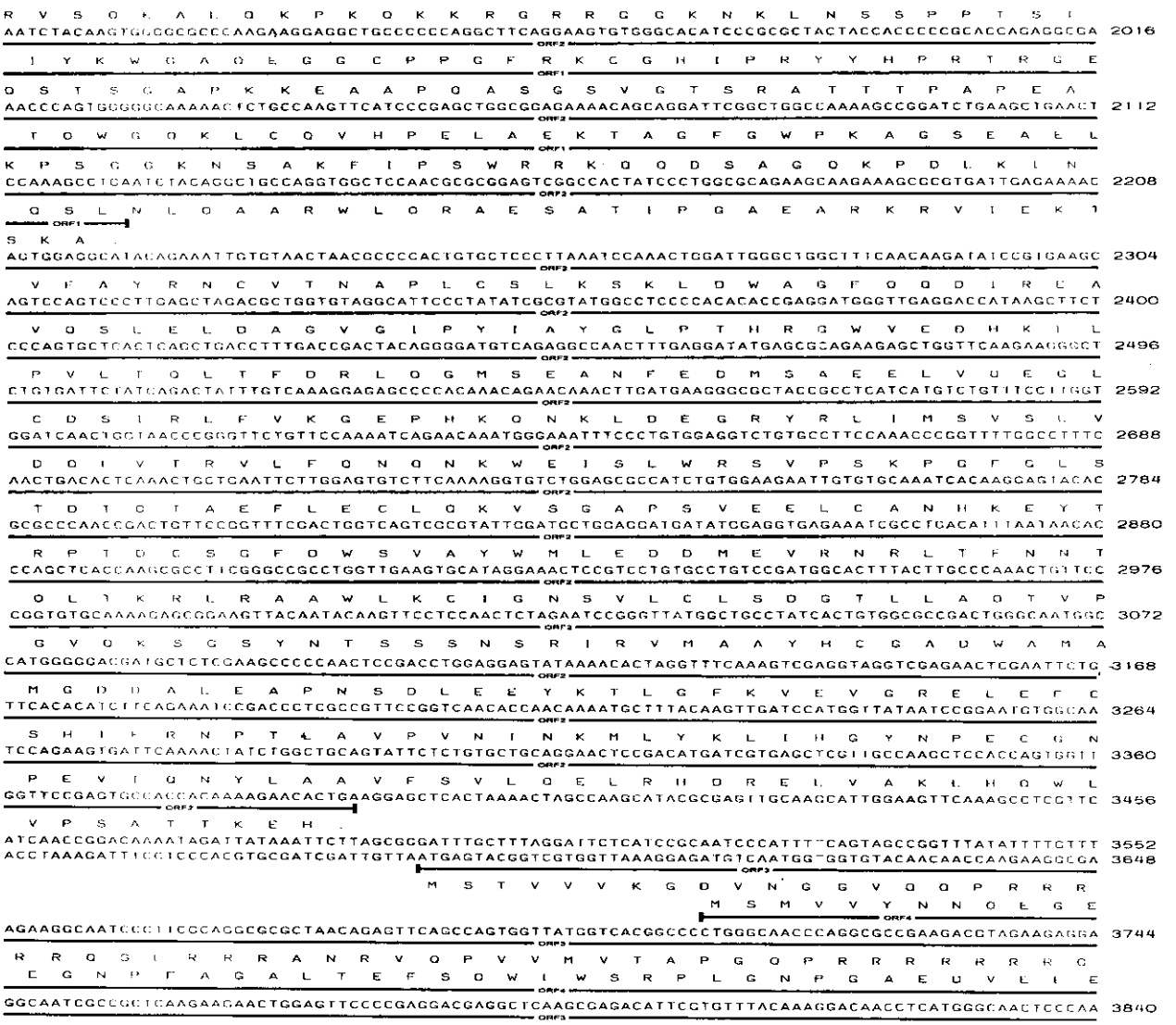
Table (1): Primers used for PCR reactions to generate PLRV-full length.

Primer number	Sequence	Position
P2	5'-cggcggtggttgggtcgtcaagtt-3'	413-390
P3	5'-CTGCGGCACCCACCCCGCTAT-3'	340-360
P4	5'-cgacgttgcgaaagcgcc-3'	995-978
P5	5'-GAGAGAACGCCTTGGTGACAG-3'	940-960
P6	5'-catcagcccagttcttgccggcaa-3'	1524-1501
P7	5'-TGAAAGAGCTAGAGCGGGAAGCAT-3'	1441-1464
P8	5'-ggtttgacctctggtgcgggg-3'	2020-2000
P9	5'-GTGGGGCGCCCAAGAAGGAGG-3'	1930-1950
P10	5'-tcccttttgttctgattttggaac-3'	2640-2617
P11	5'-TCCTTGGTGGATCAACTGGTAG-3'	2585-2606
P12	5'-tcggcgccacagtgataggca-3'	3060-3040
P13	5'-AGCGGAAGTTACAATACAAGTTCC-3'	2990-3013
P14	5'-gctagtgggttgatacccttcgca-3'	4533-4510
P15	5'-CAAGCCGTCCCTATGTATTA-3'	4450-4469
P16	5'-ctctaagttcgggtgggtgccttgg-3'	5212-5188
P17	5'-ACCCACGATGCTACAGTCGAT-3'	5140-5160
P18	5'-actacacaaccctgtaagagga-3'	5883-5862

The viral RNA indicated the presence of the 8 open reading frames (ORFs) designated as ORF0-ORF7 with molecular weights of 30.4, 69.7, 65.9, 23.3, 17.3, 56.5, 7.1 and 14.1 kDa for ORF0, ORF1, ORF2, ORF3, ORF4, ORF5, ORF6 and ORF7 respectively. These ORFs are present in all PLRV isolates (Fig.1). Function of these genes in the viral life cycle was determined elsewhere in details (Miller *et al.*, 1995; Mayo and Ziegler-Graff, 1996; Ashoub *et al*; 1998). The comparison between the open reading frames of the Egyptian isolate and the Scottish isolate of PLRV indicated that ORF1 showed the highest

degree (98.7%) of similarity. On the other hand, ORF7 showed the lowest degree (97.1%) of similarity when compared to the corresponding frames. ORF0, ORF2, ORF3, ORF4, ORF5 and ORF6 showed 98.3%, 98.5%, 97.8%, 98.3%, 97.8% and 97.4% similarity, respectively, when compared to the same frames of PLRV isolated from Scotland. Finally, this is the first information concerning PLRV genome sequences that is isolated from Egypt at the molecular level. It will be essential for generating constructs to transform potato plants to introduce resistance against PLRV.





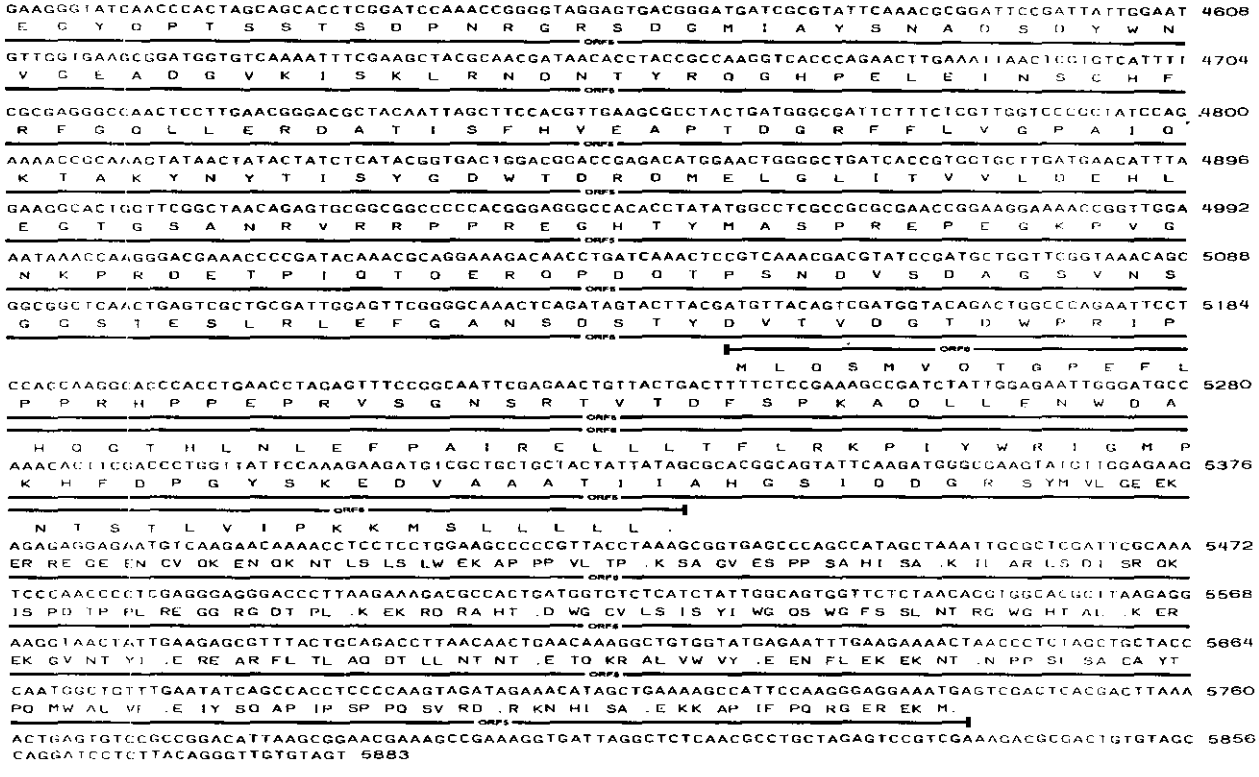


Fig. (2): Full-length sequence of PLRV genomic RNA isolated from Egypt. Open reading frames are indicated as lines. The name of each frame is determined at the middle of the line. The amino acids sequence of each frame are presented under each corresponding line.

	5	4	3	2	1	
93.3		97.9	98.1		1	1
93.3	97.7			2.0	2	2
93.3	97.8			2.1	3	3
93.3		2.2	2.4		4	4
7.1	7.1	7.1	7.1	5		5
	5	4	3	2	1	

Divergence

Egyptian isolate
Scottish isolate
Canadian isolate
Australian isolate

Fig. (3): Sequence pair distance of PLRV isolates.

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REFERENCES

Ashoub, A., Rohde, W., Prüfer, D. (1998) *In planta* transcription of a second subgenomic RNA increases the complexity of the subgroup 2 luteovirus genome. Nucleic Acids Res. **26**, 420-426

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (1994) "Current Protocols in Molecular Biology." Wiley, New York.

D´Arcy, C.J., Mayo, M.A. (1997) Proposals for changes in luteovirus taxonomy and nomeclature. Arch. Virol. **142**, 1285-1287

Eskandari, F., Sylvester, E.S., Richardson, J. (1979) Evidence for lack of propagation of potato leafroll virus in its aphid vector, *Myzus persicae*. Phytopathology **69**, 45-47

Gorbalenya, A.E., Donchenko, A.P., Blinov, V.M., Koonin, E.V. (1989) Cysteine protease of positive strand RNA viruses and chymotrypsin-like serine proteases. FEBS Lett. **243**, 103-114

Hanahan, D. (1985) In Glover, D.M. (ed.), DNA Cloning-A Practical Approach. IRL Press, Oxford, Vol.**1**, pp. 109-135

Harrison, B.D. (1984) Potato leafroll virus. Commonw. Mycol. Inst./Assoc. Appl. Biol. Descr. Plant viruses No 291. Kew, Surrey. England

Keese, P., Martin, R.R., Kawchuk, L.M., Waterhouse, L.M., Gerlach, W.L. (1990) Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll luteovirus and their relationships with two European isolates. J. Gen. Virol. **71**, 719-724

Leiser, R.M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K., Jonard, G. (1992) Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. Proc. Natl. Acad. Sci. USA **89**, 9136-9140

Mayo, M.A., Ziegler-Graff, V. (1996) Molecular biology of luteoviruses. Adv. Vir. Res. **46**, 413-460

Mayo, M.A., Barker, H., Robinson, D.J., Tamada, T., Harrison, B.D. (1982) Evidence that potato leafroll virus RNA is positive-stranded, is linked to a small protein and does not contain polyadenylate. J. Gen. Virol. **59**, 163-167

Mayo, M.A., Robinson, D. J., Jolly, C.A., Hyman, L. (1989) Nucleotide sequence of potato leafroll luteovirus RNA. J. Gen. Virol. **70**, 1037-1051

Miller, J.S., Mayo, M.A. (1991) The location of the 5`end of the potato leafroll luteovirus subgenomic coat protein mRNA. J. Gen. Virol. **72**, 2633-2638

Miller, W.A., Dinesh-Kumar, S.P., Paul, C.P. (1995) Luteovirus gene expression. Crit. Rev. Plant Sci. **14**, 179-211

Prüfer D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., Rohde, W. (1992) Ribosomal frameshifting in plants: a novel signal directs the -1 frameshift in the synthesis of the putative replicase of potato leafroll luteovirus. EMBO J. **11**, 1111-1117

Tacke, E., Prüfer D., Salamini, F., Rohde, W. (1990) Characterization of potato leafroll luteovirus subgenomic RNA: differential expression by internal translation initiation and UAG suppression. J. Gen. Virol. 71, 2265-2272

Van der Wilk, F., Houterman, P., Molthoff, J., Hans, F., Dekker, B., van den Heuvel, J., Huttinga, H., Goldbach, R. (1997a) Expression of the potato leafroll virus ORF0 induces viral disease-like symptoms in transgenic potato plants. Mol. Plant-Microbe Interact. **10**, 153-159

Van der Wilk, F., Verbeek, M., Dullemans, A.M., van den Heuvel, J.F.J.M. (1997b) The genome-linked protein of potato leafroll virus is located downstream of the putative protease domain of the ORF1 product. Virology **234**, 300-303

Van der Wilk, F., Huisman, M.J., Cornelissen, B.J.C., Huttinga, H., Goldbach, R. (1989) Nucleotide sequence and organization of potato leafroll virus genomic RNA. FEBS Lett. 245, 51-56.

الملخص العربي

قراءة التتابع النيكلوتيدى للعزلة المصرية لفيروس التفاف أوراق البطاطس

احمد عشوب

معهد بحوث الهندسة الوراثية الزراعية-مركز البحوث الزراعية-الجيزة

تم عزل فيروس أوراق البطاطس من حقول البطاطس المصرية بمنطقة كفر الزيات وتم استخدام طريقة إنزيم الانقلاب العكسي للحامض النووى الريبوزى الخاص بالفيروس والمعزول من نباتات الحرنكش البرى المصاب بالعدوى صناعيا. ثم تم إنتاج قطع متداخلة من الحامض النووى الديوكسى الريبوزى تم عمل الكلونه لنواتج التفاعل ثم قراءة التتابعات وبمقارنة العزلة المصرية مع العزالات من اسكتلندا، هولندا، كندا واستراليا اتضح وجود نسبة تشابه بدرجات ٩٣,٥،٩٧,٥،٩٧,٩،٩٨,١% تباعا وبمقارنة الإطارات المفتوحة المقروءة للعزلة المصرية والعزلة الاسكتلندية للفيروس اتضح آن إطار رقم ١ كانت الأكثر تشابها بنسبة ٩٨,٧% وكان أبعدها الإطار ٧بنسبة ٩٧،١.الإطارات ٥،٤،٣،٢،٠،و٦ قد أظهرت تقارب٩٨,٣،٩٨,٥،٩٧,٨،٩٨,٣،٩٧,٨،٩٨,٤،٩٧,٨% على التوالى بمقارنتها بالإطارات المناظرة للعزلة الاسكتلندية لفيروس التفاف أوراق البطاطس.