



Molecular, serological and biological characterizations of *Potato Leaf Roll Virus* in infected potato plants in Egypt, and its effects on plant cell organelles

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Abstract

Potato Leaf Roll Virus (PLRV) is one of the most serious viruses infecting potato plant (*Solanum tuberosum* L.) in Egypt. Indirect Enzyme Linked Immunosorbent Assay (Indirect-ELISA) results revealed that 70 % of the collected samples were infected with PLRV. A multiplex Polymerase Chain Reaction (PCR) was carried out using three different sets of primers, specific for both PLRV and *Potato virus Y* (PVY) isolates. For confirmation; the movement coat protein (MP) gene was isolated from the infected plant tissues, and a band with molecular size 336 bp was obtained using Reverse transcription-Polymerase chain reaction (RT-PCR). The DNA sequence of the Egyptian PLRV-Banha -MP gene was deposited in GenBank under an accession number of KR002119. Moreover, sequence analysis revealed that the Egyptian PLRV isolate was closely related to a New Zealand isolate of PLRV (GU002341), with identity of 100%. Transmission electron microscope (TEM) examination of PLRV showed isometric particles, with approximate size of 24-30 nm. The cytopathological examination of the potato plant infected with PLRV revealed many cellular effects such as; partially degraded and deformed chloroplast, starch with an increased size and color change, in addition to nucleus and cytoplasmic bridge. It could be concluded that PLRV is present in Egypt, infecting most of the potato cultivars. Moreover, four different strains of PLRV were detected based on the Single strand conformation polymorphism (SSCP) of the MP gene. The aims of the current study were to identify the PLRV infection of potato plant in Egypt using; molecular, serological and biological methods, in addition to studying the effect of this virus on potato cell organelles. This is the first record of the presence of four different strains of PLRV infecting potato in Egypt, using SSCP assay.

Keywords: Potato, PLRV, Indirect-ELISA, Multiplex PCR, SSCP, TEM

1. Introduction

In Egypt, yield reduction in susceptible cultivars of potato crop due to PLRV infection might reach 80-90%. In North America and Egypt; five viruses namely; *Potato leaf roll virus* (PLRV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato virus A* (PVA), and *Potato virus S* (PVS) were of primary concern, and have received considerable attention during potato seed certification programs (El-Sawy and Hadidi, 2000). Moreover; the Potato spindle tuber viroid (PSTVd) and few mycoplasma organisms could affect this crop, and cause severe damage. Salazar, (2003) reported that PLRV; PVY; PVA; PVX; PVS, and *potato virus M* (PVM) were the most important viruses worldwide, when their distribution and effects on potato yield were combined. Likewise, Valkonen, (2007) revealed that about 40 viruses were known to affect the potato crop. In the future; these viruses have the potentiality of severely limiting potato production, if their control is not considered immediately.

Positive-sense RNA viruses synthesize sgRNAs messenger RNA (mRNAs), to allow them to regulate the timing and expression levels of their downstream genes on the 3'-terminus of the viral genome. Translation of the viral replicase gene directly from gRNA was required for the synthesis of sgRNAs. These sgRNAs were synthesized shorter than their cognate gRNA, which were co-terminals either to the 5' or 3' terminals of the genomic sequence. Sztuba-Solinska *et al.* (2011) added that synthesis of these sgRNAs facilitated the translation of viral proteins i.e. structural or movement proteins that function at the intermediate and at latest ages of infection.

In eukaryotic viral systems, infection led to the induction of membranous compartments where replication occurred. Virus-encoded subunits of the replication complex mediated its interaction with the different membranes. Gushchin *et al.*, (2013) reported that as replication platforms, RNA viruses used the cytoplasmic surfaces of different membrane

compartments such as; endoplasmic reticulum (ER), Golgi apparatus, endo/ lysosomes, mitochondria, chloroplasts, and peroxisomes. In multiplex RT-PCR; three common potato-infecting viruses mainly; PLRV; PVY, and PVX, were detected simultaneously from the total RNA of potato leaves (Al-Saikhan *et al.*, 2014). The aims of this work were to identify the PLRV infecting potato cultivars in Egypt using some advanced techniques such as; RT-PCR, Multiplex-PCR, Sequencing, SSCP techniques, in addition to the use of serological and biological methods, and to study of the effect of the virus on the potato plant cell organelles.

2. Materials and methods

2.1. Virus source and symptoms

Leaves samples showing symptoms of leaves rolling; chlorosis and stunting, were separately collected from naturally infected potato plants grown at different localities of Egypt, during January-September of the 2014-2015 growing seasons (Table 1).

2.2. Serological screening for infecting PLRV in potato plant leaves

2.2.1. Indirect-ELISA

Collected leaf samples were screened using Indirect-ELISA for infection by; PLRV; PVY; PVS; PVX and *Alfalfa mosaic virus* (AMV). The used antisera were kindly prepared and provided by Prof. Gaber Fegla, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. The Indirect-ELISA was carried out according to Koenig, (1981) and modified by Fegla *et al.*, (1997).

2.3. Molecular characterization of PLRV

2.3.1. Total RNA extraction and cDNA synthesis

RNA was extracted from potato leaves infected with PLRV following the protocol described by Aseel *et al.*, (2019).

Table 1: Localities of collected potato leaf samples from different regions of Egypt, potato spp., and percentages (%) of leaves infections

No.	Localities	Region	Species	Percentage of infection (%)	Area (Feddan)
1	EL-Beheira	Komhamada (Shabour)	Spount	25	2
			Diamant	25	3
		Etay El-Baroud (El-Tawfekia)	Diamont	15	1
			Cara	20	3
2	EL-Gharbia	Kafr El-Zyate Tanta	Cara	30	3
			Cara	5	10
			Spount	5	0.5
3	Alexandria	Borg El Arab	Cara	15	1
			Diamont	15	
			Spount	5	
4	EL-Monofya	Berkt El-Saba	Diamont	20	1
		Kewesna	Cara	10	4
5	EL-Kalyobia	Banha	Spount	15	0.5
6	Matrouh	Elhamam (Kobry El-Takaml)	Spount	13	2
7	EL-Dakahlia	El-Mansoura	Spount	5	1

2.3.1. Multiplex-PCR reaction

Based on the results obtained by Indirect-ELISA, a multiplex-PCR was used to screen for mixed infections by the viral strains PLRV and PVY. This assay was optimized according to Al-Saikhan *et al.* (2014), with minor modifications. The sense and antisense primers of PLRV and PVY (Table 2) were used to amplify the CP genes of the two virus's cDNAs. The multiplex-PCR reaction was carried out by adding 1 µl aliquots of the cDNA mixture into 25 µl [containing 2.5 µl of 5 x PCR reaction green buffer; 2.5 µl of 5 x PCR reaction less buffer; 2.5 µl of 50 mM-MgCl₂; 2.5 µl of 25 mM-dNTPs; 1µl (10 pmol/µl) of each sense and antisense specific primers; 0.2 (5U/ µl) Taq polymerase (Promega, USA); and 11.8 µl of Sterile Milli Q water]. The PCR program was applied as follows; initial denaturation at 95°C for 3 min. followed by 34 cycles, each cycle consists of; 95°C for 1 min., annealing at 54°C for 1 min., extension at 72°C for 1min., with final extension step

at 72°C for 5 min. Finally, 5 µl of these PCR products were separated on agarose gel electrophoresis using 2% (w/v) agarose in 0.5x TBE buffer. Electrophoresis was carried out at 80 Volt with 0.5x TBE buffer as running buffer, and then the gel was stained with 0.5 µg/ cm³ (w/v) Ethidium bromide solution.

2.3.3. Amplification of PLRV-MP gene using RT-PCR, sequencing and phylogenetic analysis

RT-PCR components, conditions and sequencing analysis were done according to Kawanna and Aseel, (2019).

2.4. Single Strand Conformation Polymorphism (SSCP) assay of PLRV-MP genes

We expected the presence of more than one isolate of PLRV in the collected samples; so the PCR products of the MP gene were subjected to SSCP-PCR, according to Celotto and Gravely, (2001).

Table 2: The different primers used in Multiplex PCR assay

Primers	Sequences 5'-3'	Gene	Fragment	References
PLRV-CP sense PLRV-CP antisense	AGTACGGTTCGTGGTTAAAGG CTATTTGGGGTTTTGCAAAG	CP	624 bp	Presting <i>et al.</i> , (1995)
PLRV-MP sense PLRV-MP antisense	GCAATGGGGGTCCAACATCAT CGCGCTAACAGAGTTCAGCC	MP	336 bp	Awan <i>et al.</i> , (2010)
PVY-CP sense PVY-CP antisense	CAACTCCAGATGGAACAATTG CCATTCATCACAGTTGGC	CP	900 bp	Fateme <i>et al.</i> , (2012)

Ten microliters of PCR products of the PLRV-MP gene (336 bp) was combined with 2 µl of SSCP loading dye; then the samples were heated at 100°C for 5 min. to denaturize the DNA, and then chilled on ice for 5 min. After that; 10 µl of each sample was carefully loaded into the wells of the agarose gel, and then electrophoresis was applied in 1x Tris/ Borate/ EDTA (TBE) buffer using constant voltage (50v) for 4 h at 4°C. This gel was finally stained with ethidium bromide solution, and then photographed using gel documentation system.

2.5. PLRV purification and Transmission electron microscope (TEM) examination

2.5.1. PLRV purification

PLRV particles were purified from the infected potato plants according to the method of Gooding and Hebert, (1967). PLRV yield was evaluated by measuring the OD using the spectrophotometer. Moreover; the purified virus particles were examined using Transmission electron microscope (TEM) (JEOL- CX 100 operating at 80 KV), as reported by Morales *et al.*, (1990).

2.5.2. Plant cytopathological effects resulting from viral infection

Pieces of infected potato leaves which showed positive results of ELISA and PCR assays were prepared to be examined by the TEM, using standard procedures as described by Martelli and Russo, (1984).

3. Results and Discussion

3.1. Serological screening for infection of potato leaves with PLRV

3.1.1. Indirect-ELISA

Using Indirect-ELISA; five viruses were detected in the collected potato leaf samples, these viruses were; *Potato leaf roll virus* (PLRV); *Potato virus Y* (PVY); *Potato virus X* (PVX); *Potato virus S* (PVS), and *Alfalfa mosaic virus* (AMV). Based on the obtained results; the two viruses PLRV and PVY were the most frequent viruses, either alone or in combinations with the other detected viruses (Fig.1). It is well known that ELISA is the most common technique in viral detection, especially those of the plant origin. Similarly, these viruses were detected in other countries by Glais *et al.*, (2005); Agindotan *et al.*, (2007); Burhan *et al.*, (2007); Bostan and Peker, (2009); EL-Helaly *et al.*, (2012). Moreover, many investigators applied ELISA test for serological diagnosis of PLRV (Varveri, 2006); PVY (Li *et al.*, 2006), and PVX (Boukhris-Bouhachem *et al.*, 2007).

3.2. Molecular characterization of PLRV

3.2.1. Multiplex PCR

According to Hull, (1993); one of the disadvantages of serology that only 2-5% of the viral genome encodes for antigenic determinants on the surface of the CP.

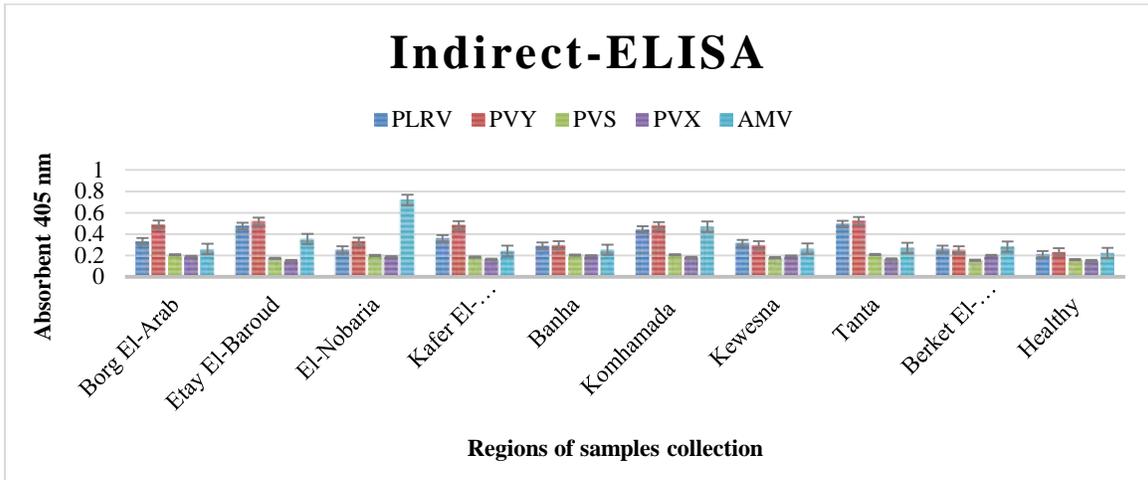


Fig. 1: Absorbance values at 405 nm of potato leaf extracts collected from different Egyptian regions, using ELISA reader

RT-PCR is known to be at least 1000 times more sensitive than ELISA, in terms of detection sensitivity of potato viruses (Leone *et al.*, 1997). Thus in order to confirm the detection of multiple viral infections in this study through Indirect-ELISA, we used Multiplex RT-PCR successfully. For potato samples infected with a single virus; the Multiplex-PCR product showed one fragment with molecular size about 624 and 900bp, for PLRV and PVY-CP genes, respectively (Fig. 2). Two fragments were observed however in

potato samples containing mixed infection with the two viruses. These results were in accordance with those of Park *et al.*, (2005); James *et al.*, (2006), who reported that mRT-PCR has a significant advantage and permits simultaneous amplifications of several viruses in a single reaction. Moreover; similar results have been reported by Peiman and Xie, (2006), who amplified the reliable regions of the viral genome of PLRV; PVY and PVX, to detect the existence of these viruses in the infected plants using multiplex RT-PCR.

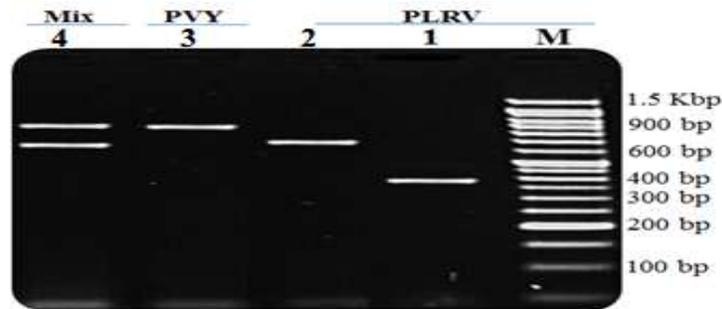


Fig. 2: Detection of multiple infections (PLRV and PVY) in some potato leaves samples using multiplex-PCR. Where; M: 1.5 Kbp DNA markers, Lane 1: PLRV- MP gene, Lane 2: PLRV-CP gene, Lane 3: PVY-CP gene, Lane 4: CP genes for both PLRV and PVY

3.2.2. Amplification of PLRV-MP gene, sequencing and phylogenetic analysis

For all PLRV isolates, PLRV-MP specific primers were succeeded to amplify one band with approximately molecular size of 336 bp (Fig. 3). In addition, the MP gene showed differentiation of gene expression in a fragment pattern band. Similarly, Hossain *et al.*, (2013) amplified and cloned 346 bp amplicon of PLRV-CP gene. The MP gene of PLRV was also aligned and compared with other *poterovirus* available in the GenBank database. Furthermore; the nucleotide DNA sequence of the MP gene of PLRV-

from Banha, (Accession number: KR002119) was closely related to *Potato leaf roll virus* of a New Zealand isolate (Accession number: GU002341), with identity of 100% (Fig. 4). The phylogenetic tree revealed that current Egyptian PLRV isolate was separated in one cluster, and was included with different viral isolates from the same origin. In agreement with Pallas and García, (2011), the variable expression of viral genes in potato host cells might be due to; time and place of sampling, relative environmental conditions, degree of infection, virus type and stage of viral life cycle, plant genotype and plant-virus interactions.

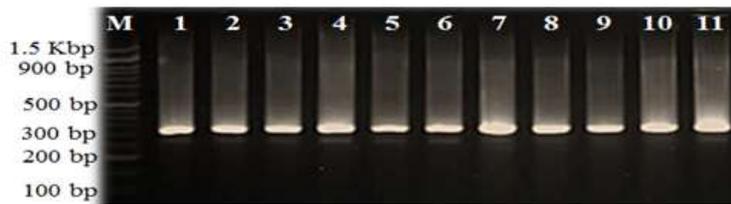


Fig. 3: 2% Agarose gel electrophoresis of PCR products of PLRV-MP gene from different Egyptian regions. Where; M: 1.5 Kbp DNA marker, Lane 1: Abise, Lane 2: Etay El-Baroud, Lane 3: Kom Hamada, Lane 4: Kafer El-Zayate, Lane 5: Tanta, Lane 6: Berket El-Sabaa, Lane 7: Kewesna, Lane 8: Banha, Lane 9: El-Bangar, Lane 10: El-Nobaria, Lane 11: Borg El-Arab

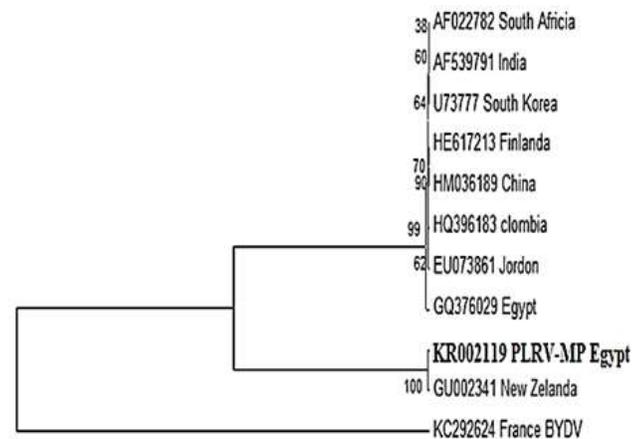


Fig. 4: Phylogenetic relationships of MP genes of an Egyptian PLRV isolate based on the DNA nucleotide sequencing, showing 100 % identity with other genes of a New Zealand isolate listed in the GenBank

3.2.3. SSCP analysis for detection of PLRV-MP gene

Single Strand Conformation Polymorphism (SSCP) analysis is one of the most widely used practical approaches for detection of one or two base pair changes in an amplified region of DNA. SSCP can aid in the identification of mutations that were selected for various bacteriological or viral systems (Morohoshi *et al.*, 1991). Moreover, it has been used to classify viral strains (Kalvatchev and Draganov, 2005; Jooste *et al.*, 2010). Orita *et al.*, (1989) previously reported that SSCP was a simple and powerful technique for identifying sequence changes in amplified DNA. For this reason; the amplicon with DNA length of about 336 bp (PLRV-MP gene) was subjected to SSCP analysis, to fingerprint the number of PLRV isolates in

the collected potato samples from different regions of Egypt. Results showed that some samples had different patterns; subsequently the PLRV isolates were divided into four groups (Fig. 5). The first group included; Etay El-Baroud; Tanta and Borg El-Arab, whereas the second group contained three isolates from Kom Hamada; Kafer El-Zayate and Kewesna. Moreover, the third groups included only one isolate from Banha, while the fourth group contained two isolates only from El-Bangar and El-Nobaria. This meant that PLRV isolates might be mutated into different strains, and each of them adapted to specific environmental conditions of the regions under study. According to Hayashi, (1992), this was probably because any slight sequence changes have their major effects on viral conformation.

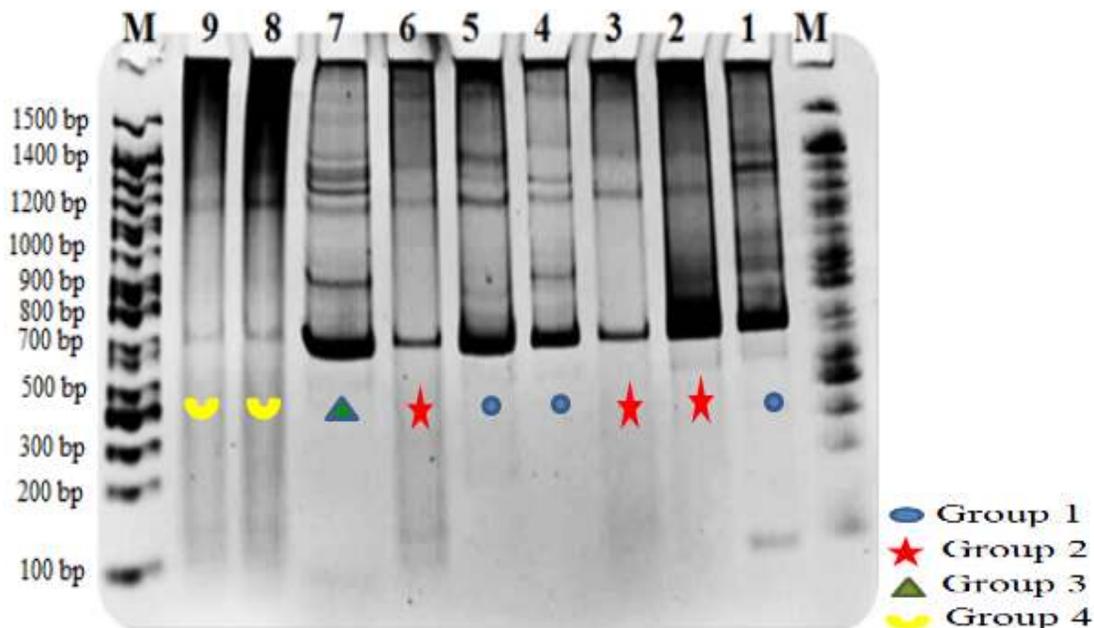


Fig. 5: Single Strand Conformation Polymorphism (SSCP) analysis of PLRV-MP gene from different regions of Egypt. Where; M: 1.5 Kbp DNA marker, Lane 1: Etay El-Baroud, Lane 2: Kom Hamada, Lane 3: Kafer El-Zayate, Lane 4: Tanta, Lane 5: Borg El-Arab, Lane 6: Kewesna, Lane 7: Banha, Lane 8: El-Bangar, Lane 9: El-Nobaria. Accordingly, the Egyptian PRRV isolates were divided into 4 groups

3.3. Transmission Electron Microscope (TEM) examination of PLRV

3.3.1. PLRV purification and TEM examination

The purity of the virus was 1.112 at absorbance range (O.D.) of 260/ 280. When the purified virus particles were examined using TEM; isometric particles were observed with approximate size of 24-30 nm. Similarly; Awasthi *et al.*, (2014) reported that isometric PLRV particles with average size of 24-25 nm, were observed in their preparation.

3.3.2. Cytopathological effects

Electronic micrographs of ultrathin sections prepared from healthy potato (*S. tuberosum* L.) leaves, showed normal cell structures including; normal chloroplast, nucleus, cytoplasm, mitochondria, and starch (Fig. 6A, B). On the other hand those prepared from PLRV-infected potato leaves revealed many cytopathological effects such as; partially degraded

and deformed chloroplast (Fig. 6C), the formation of nucleus and cytoplasmic bridge (Fig. 6D). In addition, viral-like particles with about 25 nm in diameter, inclusion bodies and ribosomes were observed as well (Fig. 6E), starch with an increased size and had change in color. Moreover; Fig. (6F and G) demonstrated the effect of infection on Plasmalemma, Parenchyma cell and Peroxisomes of plant cells. Hinrichs *et al.*, (1999) attributed these effects of viral infection on plant cells to biochemical aberrations, accompanied by pathological changes on the cellular and histological levels that become eventually visible as disease symptoms. Also, El-Deeb and IsHak, (2004) revealed histological and cytological changes in sweet potato leaves, due to the infection by *Sweet potato feathery mottle virus* (SPFMV). Similarly; using the TEM ultrathin sections, cytopathological changes were detected by Alfaro-Fernández *et al.*, (2009) on tomato leaves co-infected by *Tomato torrado virus* (ToTV) and by other viruses.

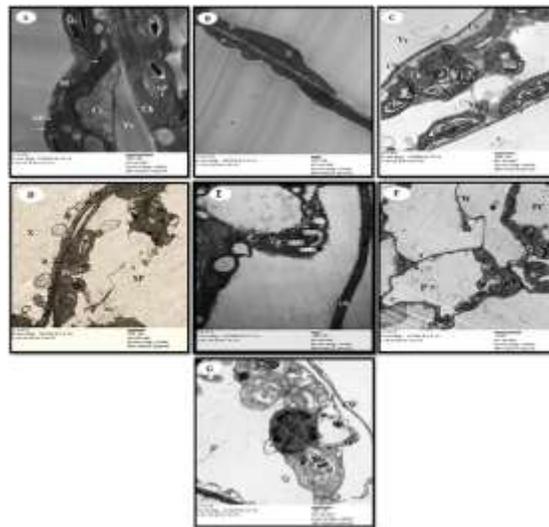


Fig. 6: Electronic micrograph of ultra-thin sections from healthy potato leaves and PLRV-infected leaves, at magnification JEOL-CX 100 of the TEM. Normal cells of healthy potato- leaves (A and B); Abnormal cells of PLRV-infected leaves (C, D, E, F and G). Where; Starch grains: (SG), Chloroplast: (Ch), Cytoplasm: (Cy), Mitochondria: (Mi), Vascular: (Vc), Nucleus: (N), Cell Wall: (CW), Inclusion bodies: (In), Viral particles: (VP), Ribosomes: (R), Plasmalemma: (P), Green plastids: (GP), Parenchyma cells: (Pc), Peroxisome: (Pr), Xylem tracheal elements: (X), Xylem parenchyma: (XP), and Grana lamella: (GL)

Conclusion

We successfully observed cytopathological effects of PLRV on potato leaves using TEM. This study presented first report of infection of potato plant by new strain of PLRV using SSCP analysis. The coding region of MP gene of this PLRV was sequenced, recorded in GenBank, and given an accession number of KR002119.

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Conflict of interest

There was no conflict of interests among the authors of this study

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