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CYTOLOGICAL, HISTOLOGICAL AND MOLECULAR CHARACTERISATION OF TWO ISOLATES OF CUCUMBER MOSAIC VIRUS (CMV) IN EGYPT

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A R T I C L E   I N F O

A B S T R A C T

Two isolates of *Cucumber mosaic virus* (CMV), CMV-wild tobacco (from Alexandria governorate) and CMV-cucumber (from Kafr El-Sheikh governorate) were investigated in this study. Cytological studies on epidermal strips of *Nicotiana glutinosa* leaves separately infected with each isolate revealed the presence of viral crystalline inclusion bodies within the infected cells. Electron microscopy of ultrathin sections of CMV infected *N. glutinosa* leaves showed significant alterations in the shape and internal structure of chloroplasts. The cell wall had serrated edges in infected cells but was more severe in cells infected with CMV-wild tobacco isolate compared to those infected with CMV-cucumber isolate. CMV-cucumber isolate was partially purified from systemically infected leaves of *N. glutinosa.* The ratio $A_{260/280}$ was 1.0 and the concentration of the virus in the preparation was estimated using an extinction coefficient of $E_{260nm}^{0.1\%,\ 1cm} = 5$. Yield of purified virus was about 2.8 mg/100 g fresh weight of infected *N. glutinosa* leaves. Electron microscopy of the purified preparation of CMV showed the presence of numerous spherical particles with a mean particle diameter of 28 nm. Amplified real-time reverse transcription-polymerase chain reaction (qRT-PCR) product of coat protein gene of each isolate was purified and sequenced. Sequences of both isolates had been submitted to GenBank Database and ware assigned accession number (LT669766) for CMV-cucumber isolate and (LT706517) for CMV-wild tobacco isolate. The sequences were edited using Chromas Pro. Version 1.34 software and compared with previously subgrouping of 27 isolates of the virus retrieved from the GenBank database. Both CMV-wild tobacco and CMV-cucumber isolates were closely related to the isolate with the accession number AJ585086 with a similarity of 97.07% and 98.54%, respectively, suggesting that the two isolates belong to subgroup II. According to the available literature, this is the first report in Egypt where CMV isolates belonging to subgroup II have been obtained.

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INTRODUCTION

Cucumber mosaic virus (CMV) is a member of the family: *Bromoviridae* and genus: *Cucumovirus* (Sastry *et al.*, 2019). CMV is one of the most economically important viruses infecting plants worldwide. The virus has probably the widest host range as it infects approximately 1200 plant species causing heavy economic losses (Zitter

and Murphy, 2009). The genome of CMV consists of three positive sense, single stranded RNAs (RNA 1, RNA 2 and RNA 3) packaged in separate particles and a subgenomic RNA (RNA 4) encoded by RNA3 that is involved in encapsidation (Suzuki *et al.*, 1991). Numerous strains of CMV have been reported from all over the world classified into two major subgroups, I and II, based on serology and

nucleotide sequence homology (Owen *et al.*, 1990; Hull, 2002; Sulistyowati *et al.*, 2004), host reaction (Daniels, 1992), and restriction fragment length polymorphism (RFLP) (Sialer *et al.*, 1999). These two subgroups, I and II, generally show about 75% sequence similarity, whereas strains in the same subgroup show 90 - 99% sequence identity (Takeshita *et al.*, 2004). CMV subgroup I has been recently divided into IA and IB on the basis of gene sequences available for CMV strains and phylogenetic analysis (Roossinck, 2002). In Egypt, almost all isolates of CMV studied so far (Sofy and Soliman, 2011; Megahed *et al.*, 2012; El-Borollosy and Waziri, 2013; Nour El-Din *et al.*, 2013; Rabie *et al.*, 2017) were found to belong to either subgroup IA or IB. According to the available literature, no work has so far been reported on the isolation of a CMV belonging to subgroup II in Egypt. The aim of the present investigation was, therefore, to find the natural variation exist among CMV isolates in Egypt and to elucidate their cytological, histological, molecular characteristics and phylogenetic analysis. Attempts were made to reveal the likely presence of CMV isolates belonging to subgroup II among those subjected to this study.

MATERIALS AND METHODS
Virus source and maintenance
Two isolates of CMV, CMV-wild tobacco isolated from Borg El-Arab (Alexandria governorate) and CMV-cucumber isolated from Desok, (Kafr El-Sheikh governorate), isolated and identified by Wagih *et al.* (2021) were maintained on *Nicotiana glutinosa* which served as a source of the virus for subsequent studies.

Mechanical inoculation
Except otherwise stated, virus inoculum of each isolate was separately prepared by grinding infected leaf tissues (1:10, w/v) in 0.01 M sodium phosphate buffer, pH 7.0 containing 0.5% 2-mercaptoethanol using sterilised and pre-chillded mortar and pestle (Dheepa and Paranjothi, 2010). Leaves of *N. glutinosa* to be inoculated were first dusted with carborundum (600 mesh) then inoculated by rubbing using forefinger with a freshly prepared inoculum. Inoculated plants were labeled and kept under glasshouse conditions.

Histological and cytological changes induced by CMV
Examination of inclusion bodies by light microscopy
Crystalline inclusion bodies (CIB) were observed in the epidermal strips from the lower surface of *N. glutinosa* leaves infected with each CMV isolates (12 days post inoculation DPI) by following standard method (El-Baz, 2004; Megahed, 2008; Taha, 2010). Corresponding samples from healthy plants were also examined as a check.

Examination of ultra-thin leaf sections by transmission electron microscopy
Three small pieces (1x1 mm each) of fresh *N. glutinosa* leaves infected with either of two isolates of CMV (15 DPI) and a healthy control plants were separately cut out and fixed under vacuum in 2% glutaraldhyde and 2% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.0) for 2 hours at room temperature, rinsed with the same buffer several times. The specimens were then post fixed in 2% Osmium tetroxide (OsO_4) in the same buffer at 4 °C for 2 hours. Samples were washed in the buffer and dehydrated at 4 °C through a graded series of ethanol and imbedded in Spurr's epoxy-resin. Ultrathin sections of 10 nm thickness were cut off by a glass knife of an LKB ultramicrotome and stained with 1% toluidine blue to select out by light microscopy the proper sections for electron microscopy. Sections were placed on grids and stained with 0.5% uranyl acetate for 15 min followed by lead citrate for 2 min. before examination by a Jeol JEM-100ex electron microscope, Japan (Martelli and Russo, 1984; Medina *et al.*, 2003).

Purification of CMV
CMV-cucumber isolate was purified from CMV-systemically infected leaves of *N. glutinosa* by the method described by Tomlinson *et al.* (1973) and applied by Ashby (1977) and Aglave *et al.* (2007) with some modifications. A portion of 100 g fresh infected leaves showing systemic symptoms were harvested 18 DPI then homogenised in ice-cold 100 mL of 0.5M potassium phosphate buffer (pH 7.5) containing 0.2 mL thioglycollic acid and 0.001M-EDTA (extraction buffer). The homogenate was squeezed and filtered through two layers of cheesecloth; the pulp scraped off the muslin was re-extracted in 50 mL of extraction buffer. An equal volume of diethyl ether (1:1, v/v) was then added to the filtrate and stirred up for 20 min, after which it was subjected to low-speed centrifugation (UNIVERSAL 32-HETTICH) at 500 x g for 15 minutes at 4 °C. The aqueous phase (Lower) was aspirated and retained for 30 min at 20 °C to remove excess ether and the pellet was discarded. The whole mixture was centrifuged

(UNIVERSAL 32-HETTICH) at 8000 x g for 30 min at 4 °C after which the pellet was discarded and the supernatant retained followed by ultra-centrifugation to precipitate the virus from the clarified extract by centrifugation (OPTIMA XE- BECKMAN COULTER) at 35,000 xg for 120 min at 4 °C. Pellets were re-suspended in 1 mL of disodium tetraborate buffer (pH 9.0) containing of 0.005 M- EDTA and the preparation was stored at 4 °C for 20 h (overnight). The suspension was then subjected to low-speed centrifugation at 500 x g for 10 min at 4 °C and the supernatant was, further, centrifuged two times at 8000 x g and 500 x g, respectively for 15 min at 4 °C. The supernatant containing the partially purified virus was withdrawn with a fine Pasteur pipette and transferred into a clean vial to be stored at -20 °C. Virus identity and concentration were checked biologically by inoculating leaves of *Chenopodium amaranticolor* for local lesion development. The UV-absorption spectrum of the purified virus preparation was spectrophotometrically performed over the range of 200-320 nm wavelengths with 10 nm intervals using an ultraviolet spectrophotometer (Thermo Scientific, VISION pro™). The parameters, A_{260}/ A_{280} as well as virus concentration were estimated. Concentration of the virus in the preparation was determined as outlined by Noordam (1973) using an extinction coefficient of $E_{260nm}^{0.1\%, 1cm} = 5$.

Electron microscopy of CMV
Formvar-coated nickel grids were floated on drops of purified CMV preparations for 5 minutes. After a rinse with distilled water, the grids were stained with 2% uranyl acetate and examined with a Jeol JEM-100ex electron microscope. Particles were examined at 60,000x direct magnification and their diameter measured.

Detection by real-time reverse transcription-polymerase chain reaction (qRT-PCR)
Total RNA was extracted from 100 mg of *N. glutinosa* leaves infected with the isolates under investigation and an equal quantity of healthy plant leaves as control using GeneJet RNA Purification Kit™ (Thermo scientific Company, Canada) according to manufacture instructions. Total RNA was used for complementary DNA (cDNA) synthesis using Rotor-Gene Q (QIGEN, Company). Coat protein (CP) gene of CMV was amplified using one step RT-PCR kit (SnsiFastTM SYBER® Lo-ROX One-Step Kit, Bioline Reagents Ltd,

USA), according to manufacturer instructions. cDNA synthesis was carried out in a total volume of 20 μL containing: 0.4 μL RiboSafe RNase Inhibitor, 1 μL each of the forward (5′- TATGATAAGAAGCTTGTTTCGCGCA-3′) and reverse primer (5′-TTTTAG CCGTAAGCTGGATGGACAACCC-3′ (Dietzgen *et al.*, 2001), 0.2 μL of reverse transcriptase, 4 μL of RNA template (about 50 ng), then the volume completed with 13.4 μL DEPC-H_2O RNase free. The reaction conditions of qRT-PCR were as follows: one cycle of 45 °C for 10 min (reverse transcription), one cycle of 95 °C for 2 min (polymerase activation), 40 cycles of 95 °C for 5 sec (denaturation), 60 °C for 10 sec (annealing) and extension at 72 °C for 5 sec. The amplification product was re-amplified by the conventional PCR to obtain high yield of cDNA. PCR reaction was carried out using a thermal cycler (Primus 25 Advanced®, Peqlab Company) in 25 μL mixture containing: 12.5 μL of Master Mix (MyTaq™ Red Mix), 0.5 μL of each primer, 3 μL of cDNA (template), 8.5 μL of sterile nuclease-free distilled water. The initial denaturation was performed at 90 °C for 45 sec, followed by 40 cycles of denaturation at 94 °C for 45 sec, annealing at 60 °C for 45 sec and extension at 72 °C for 1 min. PCR reaction was terminated by final extension at 72 °C for 10 min. The PCR products were separated in agarose gel (1.5%), stained with RedSafe™. Electrophoresis was carried out at 100 volts (Cleaver Scientific Ltd) for 20 minutes. DNA bands were visualized on a UV-transilluminator.

Molecular studies
Sequence analysis of CMV (cucumber and wild tobacco) CP gene
Nucleotide sequencing of the coat protein gene of each isolate was carried out on the corresponding purified PCR product using BigDye® Direct Sanger Sequencing Kit and sequencing product was purified using Centri-Sep spin column and resolved on an ABI PRISM® 3700 Genetic Analyzer at Sigma Scientific Services Company. The isolates were compared with the previously described sequences in the NCBI database using the BLAST program.

Sequence alignment and phylogenetic analysis
Pairwise and multiple DNA sequence alignment were carried out using ClustalW multiple sequence alignment program version 1.82 (http://www.Ebi.ac.uk/clustalw) (Thompson *et al.*, 1994). Bootstrap neighbor joining tree

was generated using MEGA programme version 6.0 (Tamura *et al.*, 2013) by applying the neighborhood Joining method and the Kimura 2-parameter model (Kimura, 1980; Shevchenko *et al.*, 2015). Sequences had been submitted to GenBank Database (CMV-cucumber, accession No. LT669766; CMV-wild tobacco has accession No. LT706517) were compared with the CP gene sequences of twenty seven CMV strains belonging to subgroup IA, IB and II obtained from GenBank Database (Verma *et al.*, 2006; Sofy and Soliman, 2011), and the similarity was calculated using the Superimposed Image Analysis System (SIAS) software programme by pairwise method (http://imed.med.ucm.es/Tools/sias.html).

RESULTS

Characterisation of two Egyptian isolates of CMV (CMV-cucumber isolate) and (CMV-wild tobacco isolate) was based mainly on the cytological, histopathological, U.V. absorption spectrum of the purified virus, qRT-PCR and phylogenetic analysis.

Histological and cytological changes induced by CMV
Careful examination of epidermal strips and ultrathin sections of *N. glutinosa* leaf tissues separately infected with either CMV-cucumber isolate, or CMV-wild tobacco isolate and phosphate buffer mock-inoculated healthy leaves, by both light microscopy and transmission electron microscopy, revealed the occurrence of a number of cytological and histopathological changes as described hereunder.

Examination of inclusion bodies by light microscopy
Epidermal strips of CMV-infected leaves of *N. glutinosa*, separately inoculated with the two test isolates, CMV-wild tobacco and CMV-cucumber, in addition to epidermal strip of phosphate buffer-inoculated leaves were prepared and stained with the mercuric bromophenol blue reagent and examined with light microscopy. Examination revealed the presence of crystalline inclusion bodies in infected cells (Figure 1). Such inclusions have never been observed in epidermal strips derived from healthy, phosphate buffer-inoculated leaves.
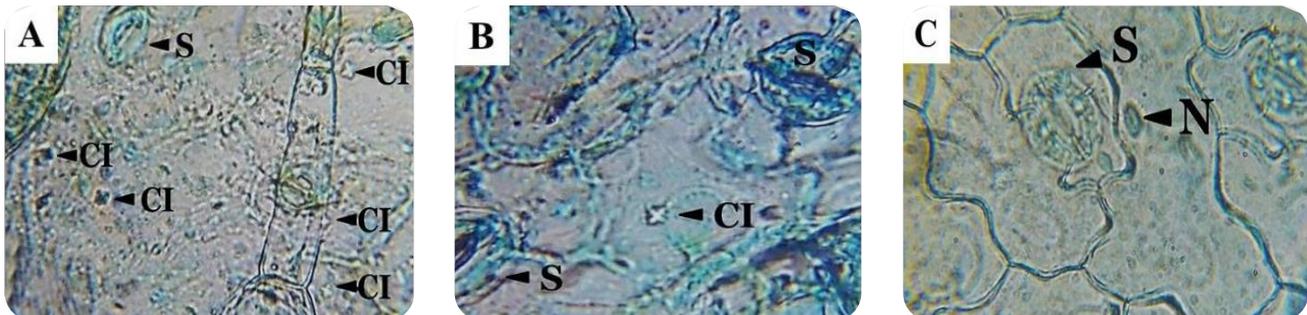

Figure 1. Light micrograph of a surface view of an epidermal strip of *N. glutinosa* infected with a CMV-wild tobacco isolate (A) and CMV-cucumber isolate (B), 15 DPI as compared to that of a healthy (control) specimen (C). Arrow heads point at crystalline inclusion bodies (CI), nucleus (N) and stomata (S). Magnification X400.

Examination of ultra-thin leaf sections by electron microscopy
Examination of ultra-thin sections of *N. glutinosa* leaves separately infected with either CMV-cucumber isolate or CMV-wild tobacco isolate and phosphate buffer mock-inoculated healthy leaves by transmission electron microscopy showed a large number of significant changes due to viral infection. In infected cells, chloroplasts assumed abnormal swollen shape, intergrana lamellae were disorganised and grana appeared loose and lacking integrity. Starch grains were either almost absent (Figure 2A) within chloroplasts from cells infected with CMV-wild tobacco isolate or reduced in size in cells infected

with the CMV-cucumber (Figure 2B).
In some cells, swollen chloroplasts seemed to have ruptured as a result of CMV-cucumber isolate infection (Figure 2C). Such alterations were never observed in phosphate buffer mock-inoculated healthy specimen (Figure 2D). As to cell wall, CMV-wild tobacco isolate caused it to become serrated at both sides (Figure 3A) whereas serration was less obvious when CMV-cucumber isolate was involved (Figure 3B). Phosphate buffer mock-inoculated healthy specimen showed no such changes (Figure 3C).

Purification and electron microscopy
Absorption spectrum of the partially purified virus
CMV-cucumber isolate was purified from *N. glutinosa*

leaves showing systemic mosaic symptoms. When absorption spectrum over the range from 200-700 nm wavelength was performed, the ultraviolet absorbance of the purified virus preparation was found to be similar to that of nucleoproteins with a minimum at 220 nm and a maximum at 265 nm. The ratio $A_{260/280}$ was 1.0. Concentration of the virus in the preparation was

estimated according to Noordam (1973) using an extinction coefficient of $E_{260nm}^{0.1\%, 1cm} = 5$. The yield of purified virus was about 2.8 mg/100g infected leaves. The partially purified preparation was tested for infectivity on *C. amaranticolor* as a diagnostic host which gave necrotic local lesions, 5 DPI.
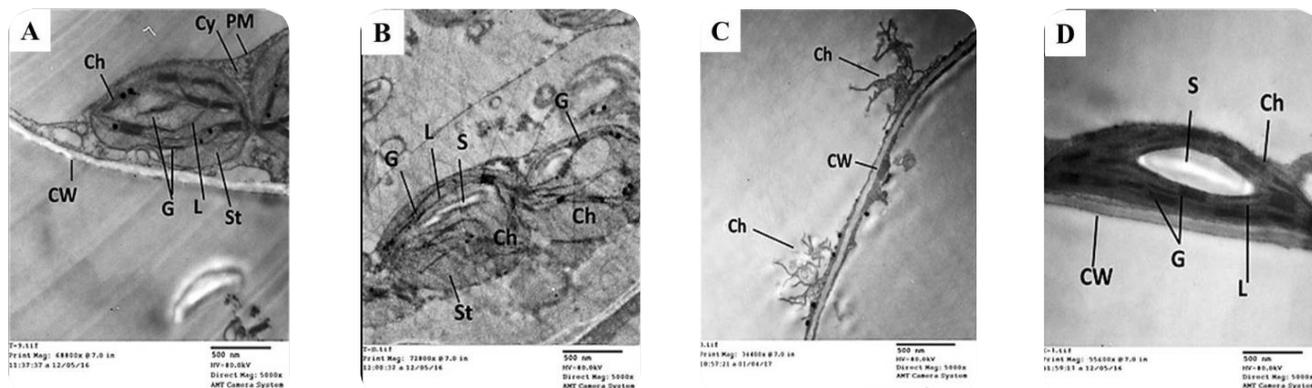

Figure 2. Transmission electron micrographs of ultra-thin sections of *N. glutinosa* leaves infected with CMV-wild tobacco isolate and CMV-cucumber isolate as compared to that of the phosphate buffer mock-inoculated leaves. In CMV-wild tobacco-infected leaves, chloroplasts were found swollen with degenerated grana and disrupted intergrana lamellae with the absence of starch grains **(A)**. With CMV-cucumber isolate similar changes were observed, albeit with less amplitude and smaller size starch grains **(B)**. Later, chloroplast membrane seemed to have ruptured and the whole structure erupted with lamellae remnants being left behind **(C)**. In phosphate buffer mock-inoculated leaf cells, chloroplasts **(D)** were seen located close to the cell wall. They were assuming elliptical shape with well-developed grana and intergrana lamellae and accommodating large starch grains. Ch, Chloroplast; CW, cell wall; CY, cytosol; G, grana; L, lamella; PM, plasma membrane; S, starch grain; St: stroma. Scale bar 500 nm.
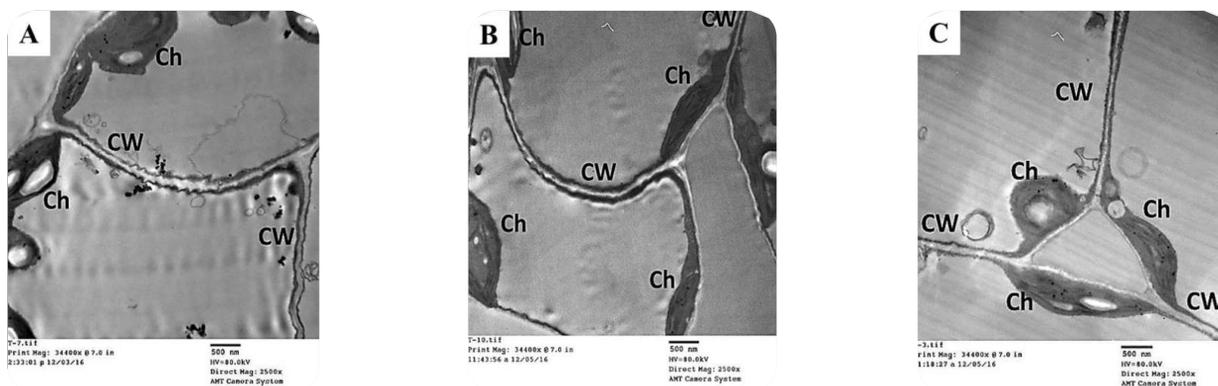

Figure 3. Transmission electron micrographs of an ultra-thin section of *N. glutinosa* leaf cells infected with the CMV-wild tobacco isolate **(A)** showing serration of both sides of cell wall and those with CMV-cucumber isolate **(B)** where serration was less pronounced. Cells of phosphate buffer mock-inoculated healthy leaves **(C)** showed no such alterations. Ch, chloroplast; CW, cell wall. Scale bar, 500 nm.

Electron microscopy of CMV

Electron micrographs of purified preparation of CMV revealed the presence of numerous isometric particles

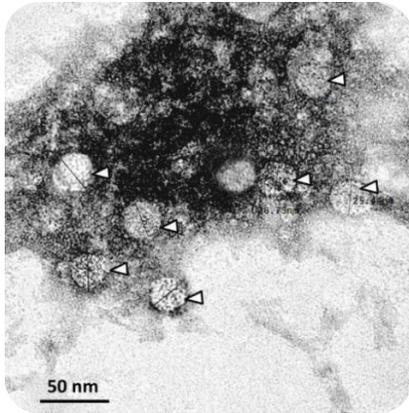(Figure 4). The modal size of particles was found to be 28 nm in diameter.

Figure 4. An electron micrograph of a partially purified virus preparation showing isometric CMV particles (pointed at by arrow heads) stained with 2% uranyl acetate and observed at a magnification of X 60,000. Scale-bar represents 50 nm.

Detection by real-time reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on total RNA extracted from *N. glutinosa* leaf samples separately infected with CMV-wild tobacco and CMV-cucumber isolates using a pair of sequence-specific primers in the coat protein gene. Analysis of the qRT-PCR result indicated that positive reaction was only obtained with each of the two samples harboring either CMV-wild tobacco or CMV-cucumber isolate and negative reaction with healthy plants, as evidenced by amplification of the target sequence as compared to phosphate buffer mock-inoculated healthy sample which gave a negative reaction as evidenced by the amplification (Green-A and Green-B) and melting curves (Figure 5).

Molecular studies
Nucleotide sequence

Nucleotide sequencing of the coat protein gene of both isolates was carried out on the corresponding purified PCR product. The nucleotide sequence of CMV-wild tobacco and CMV-cucumber coat protein gene generated were accepted in the GenBank nucleotide sequence database and given an accession number of LT706517 (CMV-wild tobacco) and LT669766 (CMV-cucumber).
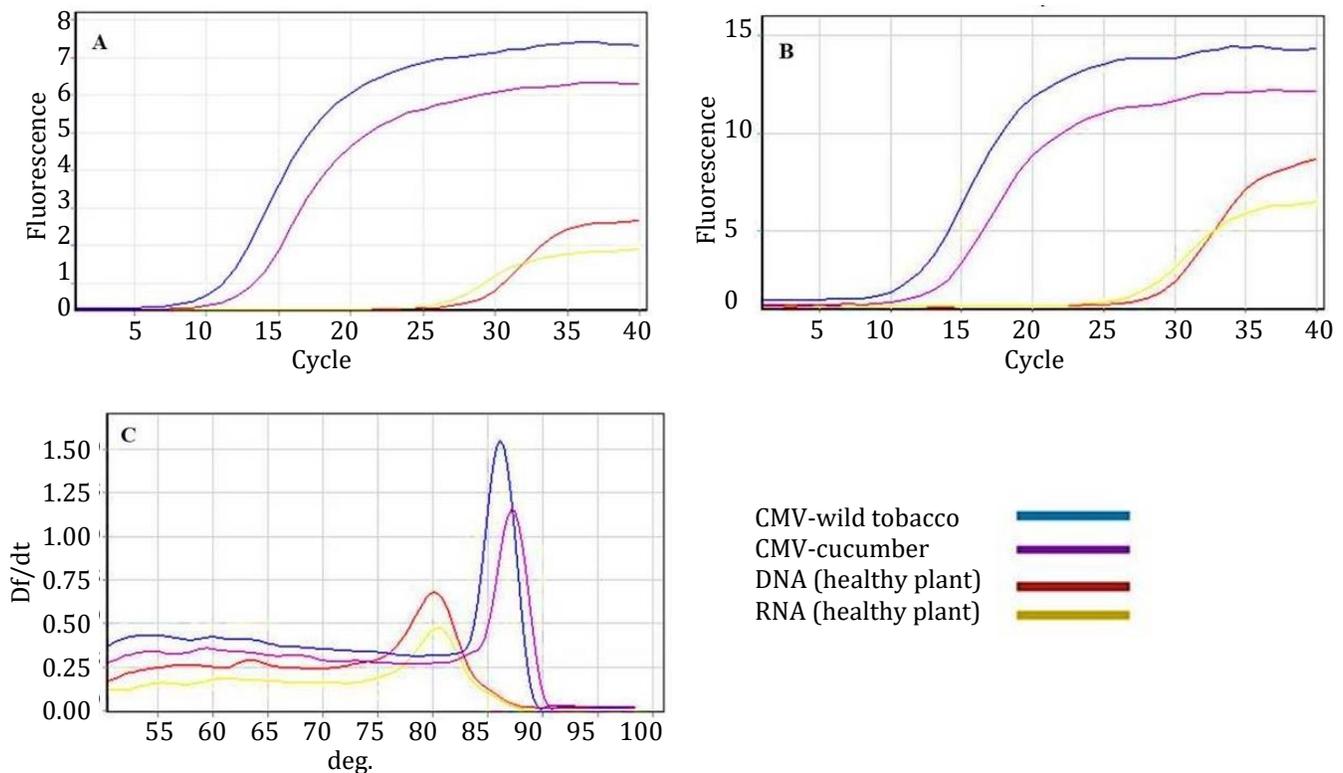

Figure 5. Standardisation of qRT-PCR for the detection of CMV, showing positive reaction with CMV-wild tobacco, CMV-cucumber and negative reaction with DNA-healthy plant and RNA-healthy plant, according to amplification curves. Green A (A); Green-B (B) and Melting curve (C).

Coat protein nucleotide sequence alignment and phylogenetic relationship analysis among CMV-wild tobacco and CMV-cucumber and several isolates of the same virus kept in GenBank

After partial sequence of CMV coat protein gene of both CMV-wild tobacco (479 nucleotide long) and CMV-cucumber (469 nucleotide long) isolates was separately determined, and sequences edited and analysed using Chromas Pro-Version 1.34 software (Seng *et al.*, 2009; Mourembou *et al.*, 2016). Sequences had been submitted to GenBank Database, accession No. LT669766 for CMV-cucumber isolate and accession No. LT706517 for CMV-wild tobacco isolate. The phylogenetic tree was developed using numerous different sequences obtained from the GenBank in order to investigate the similarity of the two isolates with others. Multiple alignment for cDNA sequences of the two CMV isolates, CMV-wild tobacco and CMV-cucumber with those of 27 CMV isolates (representing subgroup I (A and B) and II) retrieved from the GenBank made available through the NCBI was conducted using ClustalW software program version 1.82 http://www.ebi.ac.uk./clustalw/, (Thompson *et al.*, 1994). The percentage of similarity was calculated using SIAS software programme by the pairwise method and found to vary among isolates with the two isolates (CMV-wild tobacco and CMV-cucumber) investigated here being closely related and fall within subgroup II. The highest identity and similarity percentage was scored between CMV-wild tobacco (Accession number, LT706517) and CMV-cucumber (Accession number, LT669766) isolate and the Indian CMV isolate, AL (Accession number, AJ58086) with a similarity percentage of 97.07% and 98.54%, respectively. The corresponding highest similarity between CMV-wild tobacco and CMV-cucumber isolates and the American isolate, 2A1-A, (Accession number, AJ271416) belonging to subgroup IB was 77.99 and 75.19%, in the order given. In contrast, the two isolates differed in similarity with the Ban isolate (Accession number, U43888) belonging to subgroup IA, with a value of 77.49 and 76.24%, respectively (Figure 6).
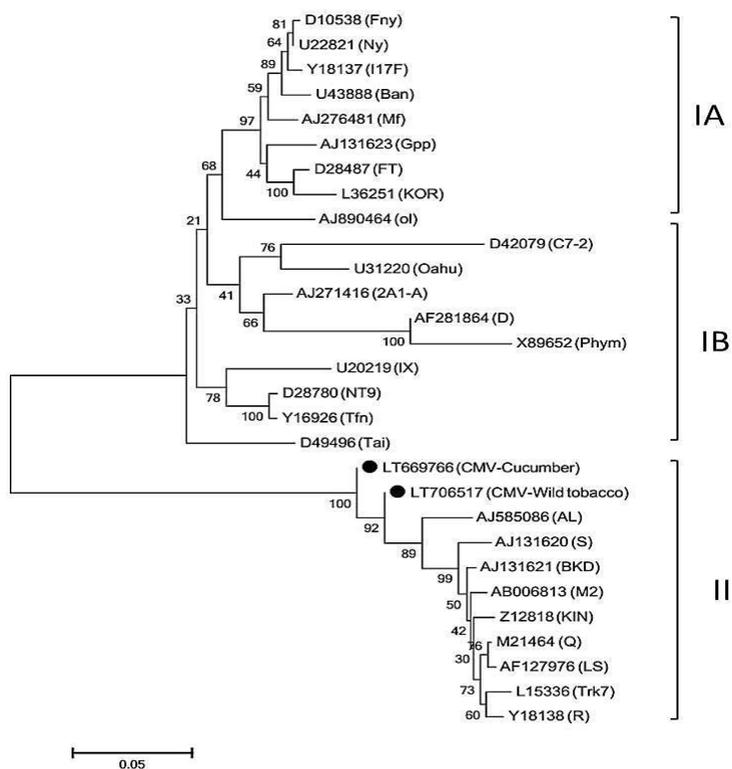

Figure 6. A phylogenetic tree demonstrating the evolutionary relationship among the two CMV isolates, CMV-wild tobacco and CMV-cucumber, investigated here and 27 different CMV entries kept in the GenBank database on the basis of partial nucleotide sequence of coat protein gene using MEGA 6. The tree was constructed using Neighborhood Joining method and Kimura 2-parameter model with bootstrap resampling of 1000 replicates. Value of bootstrap are shown next to branches.

DISCUSSION

While characterisation of CMV variations in Egypt was the objective of several investigations (Sofy and Soliman, 2011; Megahed *et al.*, 2012), it was necessary to through further light in this study on the variability of this virus. The results obtained here widens this variability on the basis of cytological, histological changes and molecular relationships.

The crystalline cytoplasmic inclusion bodies induced by the two CMV isolates and visualized in the epidermal cells of infected *N. glutinosa* leaves are identical to those reported by many other researchers (Gerola *et al.*, 1965; Honda and Matsui, 1974; Francki, 1985; Zambolim *et al.*, 1994; El-Baz, 2004; Megahed, 2008; Zitter and Murphy, 2009; Taha, 2010).

Electron microscopy has revealed that infection with CMV isolates have caused a great deal of damage at the organellar level where large number of chloroplasts showing abnormality in terms of swelling, lysis of starch grains, damage to grana and marked disorganisation of lamellae. These results were in agreement with those of Honda and Matsui (1974) and Mochizuki and Ohki (2011). CMV-wild tobacco isolate caused changes in cell wall structure with serration on both sides, while cells infected with CMV-cucumber showed crimpy shape of cell wall with less serration. No such findings have been reported before.

The partially purified virus preparation showing an absorption spectrum characteristic to a nucleoprotein and its spectrophotometrical data (A260/A280) and (Amax/Amin) obtained fell into the range previously reported for CMV by Gibbs and Harrison (1970) and Tomlinson *et al.* (1973). The slight differences observed in absorbance ratio as well as yield of virus between the present study and those reported in the literature for CMV is likely to be due to experimental errors and/or the nature of the propagative host used. The presence of numerous spherical particles of 28 nm in diameter each in the partially purified preparation as evidenced by electron microscopy correspond with those reported by other investigators (Tidona and Darai, 2001; Hull, 2002).

The CP gene of CMV isolates was successfully amplified using qRT-PCR and a total RNA preparation from the CMV propagative host *N. glutinosa* as template. When sequence alignment and phylogenetic analysis of CP gene nucleotide sequence of CMV-wild tobacco and CMV-cucumber compared with those of 27 various CMV isolates classified to CMV subgroups IA, IB and II, deposited in GenBank and identified from different locations and environments (Sofy and Soliman, 2011). The highest similarity percentage was observed between CMV-wild tobacco and CMV-cucumber isolates and the Indian isolate (AL) belonging to subgroup II. The similarity percentage revealed was 97.07% and 98.54%, respectively confirming that the two isolates belong to subgroup II. To the best of our knowledge, according to the available literature, this is the first report in Egypt where CMV isolates belonging to subgroup II have been obtained.

CONCLUSION

Characterisation of two Egyptian isolates (CMV-wild tobacco and CMV- cucumber) was carried out on the basis of cytological, histopathological, electron microscopy, shape and size of partially purified virus particles, qRT-PCR and Phylogenetic tree. Alignment of CP gene nucleotide sequence and phylogenetic relationship analysis confirmed that the two isolates (CMV-wild tobacco and CMV- cucumber) belonged to subgroup II.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

EEW proposed the scientific ideas and revised the final manuscript, EEW, MAK, MMZ designed the study. MAK, MMZ performed the experiments, data analysis, wrote the first draft of manuscript. All authors have read and approved the final version of manuscript.