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Phylogeny of Three Species Belonging to Genus Potyvirus Infecting *Solanum tuberosum* Based on Biological and Molecular Variability

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Abstract: Potato virus Y exhibits diverse strains and complexities circulating within potato fields, leading to yield deterioration. This study examined potato virus Y (PVY) isolates for their varied symptom expressions on potato plants. The infected potato plants were categorized into three distinct phenotypic expressions: venial necrosis (PVY-VN), yellow mosaic (PVY-YM), and mottling pattern (PVY-MP). They showed various biological indicators, including disease density index, pathogenicity, virus concentration via DAS-ELISA, and molecular variability through HC-PRO silencing gene accumulation via qRT-PCR. The phylogeny of the HC-PRO silencing gene revealed variations in similarity and genetic distance, corresponding to differences in the 207 nucleotides. These variations were observed among the three PVY isolates and between them and other PVY strains registered in the GenBank database. They were registered in GenBank under the accession numbers #OR804503, #PQ015611, and #PQ015612, corresponding to PVY-VN, PVY-YM, and PVY-MP, respectively. Based on the phylogeny, the three isolates belong to the NTN strain and exhibit a sensitivity range of 99% to 100% with AJ585342_United-Kingdom-strain_NTN_sequence and KY092173_Poland_strain_sequence.

1 Introduction

Potato virus Y (PVY) is considered the most prevalent and significant virus affecting potatoes (Solanum tuberosum L.) globally (Green et al 2017). It belongs to the genus *Potyvirus* within the family *Potyviridae.* PVY comprises a complex array of strains that have evolved through genetic recombination and mutations, enabling them to adapt to various host cultivars and environmental conditions. The primary strains that naturally infect potatoes are typically categorized into three groups based on their effects on the host. The PV-YO group (common strain) typically causes severe systemic symptoms, including crinkling, rugosity, or leaf drop. The PVYN group (tobacco venial necrosis strains) generally results in mild mottling across almost all potato cultivars. The PVY^{C} group (stipple streak strain) triggers a hypersensitivity reaction in many potato varieties (Moury et al 2002, Lorenzen et al 2006). Certain PVY^{NTN} strains can induce tuber symptoms in susceptible potato cultivars, beginning as swollen and raised tissue that eventually develops into the potato tuber necrotic ring spot disease (PTNRD), significantly

diminishing the tuber's market value (Gray et al 2010). The emergence of new recombinant strains has made PVY a significant concern for potato production worldwide. Sixteen recombinant PVY types have been identified, including nine common recombinants found across various geographical regions, such as PVYN:O, PVYN−Wi, PVY-NTNa, PVY-NTNb, PVY-NE11, PVY-E, and PVY-SYR-I, -II, and -III (Chikh-Ali et al 2007, Lorenzen et al 2008, Hu et al 2009, Karasev and Gray 2013). Additionally, seven rare recombinant types have been reported only a few times, including PVYN−Wi-156var, PVYN−Wi-261-4, PVY-SCRI-N, PVY-FrN, PVY-Nicola, PVY-T13, and PVY-nap (Lorenzen et al 2006, Chikh-Ali et al 2010, Karasev and Gray 2013, Schubert et al 2014, Green et al 2017). Egypt is among the leading potato exporters globally, with approximately 277,154 hectares cultivated and a total production of 4,689,647 tons.

PVY has long been recognized as a significant threat to potato cultivation in Egypt (Mahfouze and Mahfouze 2016, Elwan et al 2017), posing a serious risk to potato production by reducing both yield and quality. Although PVY has been isolated from infected potato plants (El-Absawy et al 2012), limited studies have focused on the genomic diversity of the PVY population in Egypt (Elwan et al 2017). Timely and accurate detection of PVY is crucial for managing potato viruses, and there is a strong demand for rapid and cost-effective methods of detection. Gathering precise data on the occurrence and prevalence of various PVY strains and genotypes is essential for evaluating potential economic losses due to infections. Real-time PCR (RT-PCR) is a reliable and rapid method for identifying and quantifying plant pathogens, as well as for detecting the expression levels of induced systemic resistance genes and defense genes in plants (Toyota et al 2008, Yan et al 2012). This study aims to explore the biological and molecular variability of three species belonging to the genus *Potyvirus* that infects Solanum tuberosum, with a focus on the phylogeny of Potato Virus Y isolates from Egypt.

2 Materials and Methods

This study was conducted from 2021 to 2023. The incidence of infected potato plants was recorded during the 2021-2023 seasons. Infected potato plants exhibiting distantly potyvirus-like symptoms were collected from open fields during various seasons in Egypt and tested serologically using a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to the method described by Clark and Adams (1977), with specific polyclonal antibody kits (LOEWE Biochemica GmbH, Germany). These plants were categorized into three groups: venial necrosis (PVY-VN), yellow mosaic (PVY-YM), and mottling pattern (PVY-MP).

The PVY-free potato tubers cv. spounta were obtained from the Brown rot project, Department of Vegetative Crops Institute, Center of Agriculture Research, Dokki, Giza, Egypt)

2.1 Isolation of PVY isolates

The infectious sap of each category of symptoms (PVY-VN, PVY-YM and PVY-MP) was extracted from the diseased leaf symptoms and mechanically inoculated into virus-free seedlings of *Datora metal L* and *Nicotiana tabacum cv.* white burly under greenhouse conditions (Virology Lab and greenhouse, Dept. of Agriculture Microbiology). The developed symptoms were recorded and confirmed serologically by DAS-ELISA.

2.1 Biological diversity of PVY isolates

2.1.1 Phenotype symptom expressions on potato plants induced by PVY isolates.

The sap from potato leaves exhibiting three categories of symptoms (PVY-VN, PVY-YM, and PVY-MP) was prepared by grinding each symptomatic leaf in a pre-chilled pestle and mortar with 0.1 M potassium phosphate buffer (pH 7.2). The resulting infectious sap was filtered through double-layered muslin cloth. Carborundum powder (600 mesh size) was uniformly dusted on the upper side of virus-free, two-week-old potato (Spunta) leaves before inoculation. The mechanically inoculated leaves were washed with a washing bottle after 2-3 minutes to remove any excess inoculum and extraneous particles. Additional healthy Spunta leaves were kept as a negative control. The inoculated plants were maintained in an insect-proof net house under greenhouse conditions. Symptoms were observed daily following virus inoculation, and the virus was detected using an ELISA test.

2.1.2 Response of PVY isolates to differential hosts

The pathogenicity assay of the three categories of symptoms (PVY-VN, PVY-YM, and PVY-MP) was determined by mechanically inoculating infectious sap onto the leaves of the differential hosts. One set of differential hosts was mock-inoculated with buffer as a negative control and then placed in an insect-proof net

house under greenhouse conditions. The symptoms were observed daily after virus inoculation, and the virus was detected using an ELISA test.

2.1.3 Determination of disease severity index of PVY isolates

The severity of the three symptom categories (PVY-VN, PVY-YM, and PVY-MP) was assessed through visual observation and a rating scale: 0 = no symptoms, 2 = vein clearing, 4 = mosaic, 6 = beginning of necrosis, 8 = 50% necrosis + 50% mosaic, and 10 = apical necrosis. The disease severity index (DSI) values were calculated using the formula provided by Yang (1996).

$$\mathbf{DSI}(\%) = \frac{\Sigma(\textbf{disease grade} \times \textbf{No. of plants in each grade})}{(\textbf{total No . of plants} \times \textbf{highest disease grade})} \times 100$$

Virus concentration was determined as an antigen at the wavelength 405 nm by DAS-ELISA.

2.2 Molecular characters of PVY

2.2.1 Extraction of total RNA

Total RNAs of the three categories of symptom plants (PVY-VN, PVY-YM and PVY-MP) were extracted from the leaves midribs and petioles of (200 mg) using an EZ-10 Spin column (Qiagen DNeasy Plant Mini Kit).

2.2.2 Nanodrop Analysis

The prepared RNAs were quantified for purity and yield across the three symptom categories in potato plants using a NanoDrop 2000 spectrophotometer (Thermo, USA) at wavelengths of 260 and 280 nm. One µl of each RNA sample extracted from infected potato leaves was analyzed to assess the quality and quantity of RNA (Haikonen et al 2013). RNA samples with sufficiently high concentrations were chosen for dilution and subsequent RT-PCR with the Custom TaqMan Gene Expression Assay (Elwan et al 2021).

2.2.3 Designing of HC-PRO silencing gene primers

Design of primer sequences: The Forward primer (FP) and reverse primer (RP) for the helper component gene (HC-PRO) were designed. PVY BLAST Gene Bank coordinates were recorded using the Geographical Positioning System (GPS). Positive bulk samples were tested individually using specific primers **(Table 1).**

2.2.4 Reverse transcriptase Polymerase chain reaction (RT-PCR)

Suspected potato plants exhibiting different symptoms (PVY-VN, PVY-YM, and PVY-MP) were tested using a Verso TM one-step RT-PCR kit (Thermo Scientific) to detect PVY with specifically designed primers for the HC-silencing gene. Total RNA extracted from the infected plants served as the template for RT-PCR amplification reactions using the VersoTM one-step RT-PCR kit (Thermo Scientific). The reaction was conducted in a total volume of 25 µl, comprising 4.75 µl of nuclease-free water, 3 µl of total RNA extract (10 ng/µl), 12.5 µl of one-step RT-PCR master mix (2x), 1.5 µl of 10 µM of each primer, 0.5 µl of Verso RT-enzyme mix, and 1.25 µl of RT-Enhancer. The amplification reaction was performed using a thermal cycler (T-Gradient Biometra, Germany) for 35 cycles, with each cycle consisting of denaturation at 95 ºC for 3 minutes, annealing at 68 ºC for 30 seconds (as specified by the designed primers), and extension at 75 ºC for 1 minute. The one-step RT-reaction is initiated with an incubation at 50 ºC for 15 minutes, followed by denaturation at 95 ºC for 2 minutes to convert RNA into cDNA.

2.2.5 Virus accumulation and expression of HC-PRO silencing gene

The variation in the accumulation and expression levels of the PVY-HC-PRO silencing gene was evaluated in potatoes infected with PVY isolates (PVY-VN, PVY-YM, and PVY-MP) through quantitative RT-PCR (qRT-PCR) and compared to healthy plants. In this regard, the expression of the PVY-CP gene was assessed using cDNA as a template. The RNAs were quantified in a single-step assay after normalizing specific concentrations with a 2 × SYBER Green qRT-PCR Master Mix kit. For cDNA synthesis, total RNA was extracted from potato leaves (three leaves per plant, in five replicates) at 20 days post-infection (dpi) using an RNeasy Plant Mini Kit (QIAGEN, Germany). As mentioned, cDNA was synthesized from each DNase-treated RNA extract (1 g) via a reverse transcription reaction and stored at -20°C for future use. Amplification reactions were conducted using a Thermo Rotor-Gene Q 560-system (Qiagen, Germany), incorporating 1.5 µl (0.1 mM) of each primer (forward and reverse) (Metabion International AG, Germany), 1 µl (10 ng) of template RNA, 8.5 µl of distilled sterile water, and 12.5 µl of qPCR SYBER Green

Table 1. Design primer sequences (Forward primer PF & Reverse primer PR) for PVY BLAST GenBank

Annulling (Tm)	HC silencing gene Primers		
	Type	sequence 5′ → 3′	Expected size
70°C	HC-1	PF-1 (5'TCTAGAGGCGCGCCGAGAAACAGCAAGCACCGTT'3)	244 bp
		PR-1(5'GGATCCATTTAAATCGCACGACAAATACAGATTCCA'3)	
65°C	HC-2	PF-2 (5'ATATTCTAGAGGCGCGCCAACCGGTGGACCTGAATCTC'3)	301 bp
		PR-2 (5'ATATGGATCCATTTAAATACAGATTCCAGTTTGCCTTGG'3)	
69°C	HC-3	PF-3 (5, ATATTCTAGAGGCGCGCCACAGCTCATGAATGGCAGGTA,3	283 bp
		PR-3 (5, ATATGGATCCATTTAAATTGAGAGCTTCCTTGTTCCACTT,3)	
68.4°C	HC-4	PF-4 (5, ATATTCTAGAGGCGCGCCATGAAATCCGCAAGCATCCAA,3)	207 bp
		PR-3 (5, ATATGGATCCATTTAAATCCTTTGCATCCTCCTCGCTA,3)	

Master Mix (2×) (Applied Biotechnology, Egypt) to achieve a final volume of 25 µl. These reactions were performed in a 36-well Thermo Rotor-GeneQ qRT-PCR system. The manufacturer's recommended universal thermal protocol includes a three-minute activation of thermo-start at 95°C, followed by 15 seconds at 95°C for initial template denaturation, 45 cycles of 60°C for 30 seconds each, and a combined annealing/extension phase at 72°C for 30 seconds. The biological repetition of qRT-PCR samples was conducted twice. The expression of the actin housekeeping gene was used to normalize the expression of the gene under study, while the relative expression of the genes was calculated using the 2-ΔΔCT method (Basma et al 2024).

Data analysis: The amplification curves of each reaction were created using sequence detection software. Additionally, an automatic setting calculated the threshold cycle number (Ct) based on the baseline. A dissociation curve was made after each reaction to distinguish treatment amplicons. Delta–delta threshold cycle (ΔΔCq) expression values were calculated for RNA samples of each treatment to determine gene expression using β-actin (reference gene) and other defense genes. The used equations are ΔCq = Cq – reference gene, ΔΔCq = ΔCq – control, and ΔΔCq expression = 2^(−ΔΔCq).

The equations represent the mathematical model of the relative expression ratio for RT-PCR (Schmittgen and Livak 2008). Additionally, they indicate the ratio of the targeted gene in samples compared to the control, utilizing the housekeeping gene included in each experiment with RT-PCR.

3 Results and Discussion

3.1 Detection of PVY in infected potato plants grown in open fields

PVY was detected in potato plants collected from various open fields between 2021 and 2023, based on external symptom expressions such as linear discontinuous mottling, inter-venial necrosis, and mosaic patterns with extreme distortion and reduction of leaf lamina (Elwan et al 2021) (**Fig 1**). Additionally, virus antigen concentrations were assayed using polyclonal antibodies specific for PVY with DAS-ELISA. Based on these phenotypes, the expression symptoms exhibited different patterns of mosaic, linear discoloration on the midrib, petiole, and varying degrees of leaf deformation induced by PVY. The observed PVY symptoms allowed for the classification into three PVY isolates: venial necrosis (PVY-VN), yellow mosaic (PVY-YM), and mottling pattern (PVY-MP). These three PVY isolates differed in antigen accumulations at 405 nm, with optical densities of 0.322, 0.375, and 0.430, respectively (**Table 2**).

Rising temperatures, relative humidity, and carbon dioxide levels are key indicators of climate change that impact water availability and the uptake of dissolved nutrients. Climate change also influences the epidemiology of plant viruses and can indirectly affect their replication and activity in host plants (Elwan et al 2017). The change in temperature primarily depends on climatic conditions, sampling times, and the number of sunshine hours. Both direct and indirect predicted climatic changes will influence the survival, distribution, virulence, pathogenicity, and vectors of plant viruses

Fig 1. Categories of naturally infected potato plants collected from various fields based on phenotype expression symptoms

Table 2. Different isolates of PVY were collected on the basis of the external symptoms

PVY Isolates	Symptom	Reaction to PVY antigen (OD Value at 405 nm)
Venial necrosis (PVY-VN)	LDCL and IC	+ 0.322 b
Yellow mosaic (PVY-YM)	LDCL, IC and R	++ 0.375 b
Mottling pattern (PVY-MP)	MD	+++ 0.430 c
Positive control	CL, MD and R	+++ 0.453 c
Negative control	-	- 0.085 a

Venial necrosis (PVY-VN): Vein necrosis (VN) and necrotic spots (NS) on the underside of the leaflets. leaves brittle and dry, (BD) some vein deepening (VD).
Yellow mosaic (PVY-YM): Leaf rugosity (LR) , vein yellow, (VY) leaf size reduction (LS) , shiny appearance and mottling (MP) associated with pendulous growth habit and stunting of the plants
Mottling pattern (PVY-MP): Severe mosaic (SM)alternating patches of light green and dark green areas (PGD) , leaf rugosity (LR). leaf top enation (LTE), size reduction with shiny appearance (LSRS)
Reaction to PVY antigen (OD Value at 405 nm) by DAS-ELISA

across many geographic regions, thereby increasing the frequency and scale of disease outbreaks. Undoubtedly, climate change driven by human activities has progressively contributed to the global epidemiology of plant viruses. NGS has become the gold standard for metagenomics and can be employed to identify novel virus species by utilizing various techniques to enrich viral nucleic acids, including the isolation of specific forms of RNA (dsRNA, siRNA, ssRNA) or virus particles. Each method has its advantages and disadvantages (Stobbe and Roossinck 2014). NGS can be applied for virus discovery, plant virus diagnostics, and studies of population diversity in individual virus strains. This deep sequencing allows for the identification of all minor variants present in a given infection. The current work explores the diversity of plant viruses not only within species but also among species or quasispecies (Stobbe et al 2013). This variation arises from multiple sources, including high mutation rates in RNA viruses,

recombination, and reassortment. The observed variation within a single plant host has a profound impact on the virus's response to selective pressures associated with new hosts, as well as factors such as bottleneck events that occur during cell-to-cell movement or vector transmission. Furthermore, with the increasing understanding of virus diversity and ongoing technological advances, questions regarding the deep evolutionary history of viruses and their relationships with hosts can now be addressed.

3.2 Biological properties

3.2.1 Phenotype symptom expression of PVY isolates

In greenhouse experiments, it was found that the three infectious saps of the three symptom categories PVY-VN, PVY-YM and PVY-MP induced different phenotypic expression symptoms, as shown in **Figs 2-3.** They showed external symptoms on inoculated potato plants cv. Spunta, such as severe mosaic, severe mottling associated with necrosis, mild necrosis, severe necrosis, top necrosis, and yellow and brown necrosis (Massart et al 2014). The three infectious saps-inoculated potato plants developed distinct symptoms appearing by 16, 20 and 22 dpi of PVY-VN, PVY-YM and PVY-MP isolated related to PVY wild strain 21 dpi as follows: (1) PVY-VN (Vein necrosis, necrotic spots on the underside of the leaflets and some vein deepening), (2) PVY-YM (Leaf rugosity, veinal yellow, vein necrosis, size reduction, shiny appearance and leaf brittle and dryness), and (3) PVY-MP (mosaic alternating patches of light green and dark green areas, leaf rugosity, necrosis, leaf top nation, size reduction with a shiny appearance and mottling associated with pendulous growth habit and stunting of the plants).

3.2.2 Disease severity of PVY isolates

The severity and infectivity of PVY-VN, PVY-YM, and PVY-MP isolates were assessed on potato plants of the cv. Spunta. The notable variability in PVY isolate infections was 90%, 88%, and 92%, compared to the PVY wild strain at 94%. The disease severity recorded was 93.43%, 90.75%, and 86.87%, while the virus concentration of the PVY wild strain was 89.25% at 0.492, 0.485, and 0.425 OD, respectively, in comparison to the PVY wild strain at 0.524 OD (**Table 3**).

3.2.3 Pathogenicity assay of PVY isolates

In the current study, the three virus isolates (PVY-VN, PVY-YM and PVY-MP) induced different phenotype expression symptoms at different incubation periods and virus concentrations in differential hosts under greenhouse conditions (Chikh-Ali et al 2010). They exhibited a variable symptom reaction; PVY-MP reacted with a local chlorotic lesion on Chenopodium amaranth color, with a virus concentration of 0.365 OD at seven days post-inoculation (dpi). In contrast, PVY-VN and PVY-YM did not show symptoms of localized lesions. The three isolates reacted with Datura metel, exhibiting severe dwarfing (PVY-VN), severe mottling (PVY-YM), and severe mosaic (PVY-MP), with virus concentrations of 0.453, 0.521 and 0.453 OD at 16, 18 and 22 dpi, respectively. On *Solanum tuberosum,* they showed crinkle, malformation and dwarfing (PVY-VN), severe mosaic and blasters (PVY-YM), and severe mosaic (PVY-3) with virus concentrations 0.321, 0.498 and 0.467 OD at 16, 20 and 22 dpi, respectively. On *N. tabacum* cv. white burly, they showed veinal necrosis (PVY-1), severe mosaic (PVY-2), and Leaf rugosity and mottling (PVY-3), which appeared with virus concentrations 0.349, 0.456 and 0.378 OD at 15, 18 and 20 dpi, respectively **(Table 4).**

The PVY was detected in collected potato plants (2021-2023) from various fields based on PVY-specific distinct symptoms and virus antigen concentration, using polyclonal antibodies specific to PVY by DAS-ELISA (Clark and Adams 1977). Based on these phenotypes and expression symptoms on potato plants at the insect-proof net house, PVY isolates were divided into three groups, namely Venial necrosis (PVY-YN), Yellow mosaic (PVY-YM) and Mottling pattern (PVY-MP) with PVY antigen concentration (0.322,0.375 and 0.430) OD at 405 nm, respectively. Combining biological and molecular diagnoses is preferred to obtain a complete and clear understanding of viral etiology in complex cases (Green et al 2017). Their comparison showed a closer degree of relatedness to isolates PVY^{ntn}, PVY^{C}, and PVYO, according to the NCPI. Phylogeny analysis revealed a high degree of relatedness to isolates from specific locations, which may indicate the probable origin of the isolates. To our knowledge, this is the first report on PVY isolates from the PVYntn group in Egypt. The three PVY isolates related to potatoes are: PVYO group, which induces severe systemic symptoms of mottling, crinkle, and rugosity; and PVYNTN group (potato veinal necrosis strains), found in almost all potato cultivars. The mechanical transmission by the leaf rub method has been attributed to the production of tannins and phenols

Fig 2. Photographs of inoculated potato plants cv Spunta with the three PVY isolates under greenhouse conditions. Disease severe indexing (DSI): Mo= leaf mottling, sM =Severe mosaic, VE =Venial enation, LN = leaf narrow, VN = Venial necrosis, LW = Leaf wilt, LY =Leaf yellow, LB = Leaf brown, YBL =Yellow brown leaf, LE =leaf epinasty, LR = Leaf rugosity

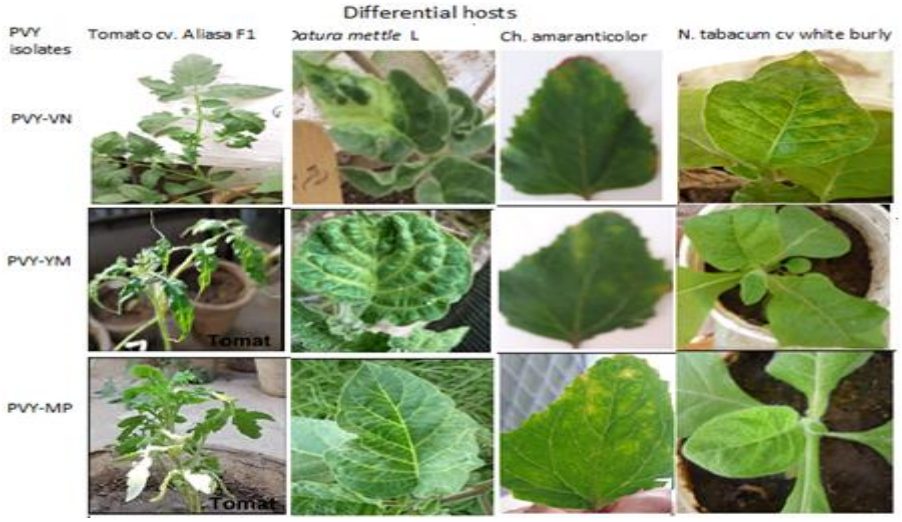

Fig 3. Photos of differential hosts inoculated with infectious sap of PVY-VN, PVY-YM, and PVY-MP under greenhouse conditions

Table 3. Disease severity and accumulation levels of PVY-HC-PRO gene in potato plants at 21 dpi

PVY isolates	IP (dpi)	% of infection (n=20)		Disease Severity		**Virus conc. OD at 405 nm.	
		(%)	% RV	(%) Value	% RV	(%) Value	% RV
PVY -strain	21 c	94	-	89.25 a	0.0	0. 524 e	0.0
Veinal necrosis (PVY-VN)	16 a	90	- 4.25	93.43 d	+4.68	0. 492 d	- 6.11
Yellow mosaic (PVY-YM)	18 b	88	- 6.38	90.75 c	+1.68	0.485 b	-7.44
Mottling pattern (PVY-MP)	22 c	92	- 2.13	86.87 b	-2.66	0.425 c	-18.89

IP = Incubation period, RV = Relative variability,
**Virus concentration = ELISA at 405 nm, +Ve = 0.546, -Ve = 0.112
The average values of the columns with the same letter do not differ significantly.

Table 4. Differential host reactions of PVY disease symptoms by the infectious sap mechanical transmitted

PVY Isolates / Differential hosts	Veinal necrosis (PVY-YN)			Yellow mosaic (PVY-YM)			Mottling pattern (PVY-MP)		
	Symptoms	VC	IP	Symptoms	VC	IP	Symptoms	VC	IP
Ch. amaranticolor L	–	0.032	-	-	0.026	-	LchL	0.365	7
D. metal L	S-Mo and Df	0.453	18	Mo& D	0.521	22	SM	0.453	25
L.esculantum cv.elisa	Cr, Ma and Dw	0.321	16	SM, B	0.498	20	SM	0.467	22
N.tabacum cv. white burly	VN	0.349	15	SM	0.456	18	Lr and Mo	0.378	20

Symptoms index, LchL = Local chlorotic lesion, S-Mo andDf = Severe mottling and dwarfing, Mo & D = mottling and dwarfing, SM, B = Severe mosaic and blasters, Cr, Ma, Dw = crinkle, malformation & dwarfing M = mosaic VN= veinal necrosis, Lr& Mo =Leaf rugosity and mottling

*VC = Virus concentration by ELISA at 405 nm

* IP = Incubation period (Days)

in the inoculated test plants due to the injury caused during the process, which activates the host response at the molecular level to synthesize the overall general chemical substance to act as a defense against any foreign substance (Basma et al 2024). The salient symptoms recorded were different patterns of mosaic, l inear discoloration on the midrib and petiole and various degrees of leaf deformation. A similar type of symptom was also reported by Ayo-John and Hughes (2014); it is one of the early reports of the disease. Subsequently, other researchers have also reported similar symptoms in leaf lamina, such as yellow streak-like mosaic patterns, reduction in size, inward curling of the leaf lamina and necrosis of leaves.

3.3 HC-PRO silencing gene detection

3.3.1 Nanodrop analysis

The total RNA of infected potato leaves with the three PVY isolates was subjected to RNA extraction using the Qiagen kit and measured by nano-drop. The total RNA yield was 215.6, 220.5 and 264.6 ng/µL, with 1.8, 1.9, and 2.1 OD RNA impurities for PVY—VN, PVY-YM and PVY-MP isolates, respectively (**Table 5**).

3.3.2 HC-PRO silencing gene amplification

The HC-PRO gene silencing of the three isolates, PVY-VN, PVY-YM, and PVY-MP, was detected in infected potato leaves using the two designed primers, HC-2 (301 bp) and HC-4 (207 bp). The gene was amplified and visualized by 1% agarose gel electrophoresis (**Fig 4**).

For the three isolates, the expected size fragments were 307 and 207 bp, respectively. The cDNA of three PVY isolates had detectable levels of 12, 15, and 25 ng/g, respectively (**Table 6).**

The identification based on HC silencing gene sequencing provided further details for specific phylogenetic levels and comparative sequence analysis. The biodiversity estimation was influenced at various levels, correlating with environmental conditions and interpreted through biological characteristics, including the phenotype expression of potato plants induced by PVY isolates, disease severity, infectivity, pathogenicity, and molecular perspectives of PVY isolates. The sequence of a highly conserved gene region, including silencing gene data, aids in predicting the correct taxonomy. Our current study aimed to sequence HC silencing genes using PCR amplification for the identification and genetic confirmation of PVY isolated from potatoes (Valli et al 2018).

In this investigation, three PVY isolates were isolated from different sites in Egypt. RNA was extracted and amplified by RT-PCR, a conventional method that utilizes specific primer sequences for the gene encoding HC for silencing. Several authors have been using the HC silencing gene sequence to investigate genetic variability (Valli et al 2018).

3.4 Genetic variability analysis of PVY isolates

3.4.1 Phylogenetic tree

Based on MSA analysis, the phylogenetic tree was constructed, revealing three clusters. In one cluster, PVY-VN, PVY-MP, and PVY-YM were found to be highly homologous with strain PVYNTN #AJ585342 and #KY092173 at 98% and 99%, respectively, with a genetic distance of 0.021. Additionally, PVY-VN, PVY-YM, and PVY-MP were homologous with strain PVYo#AY547324 and #KY112748 at 96%, resulting in a genetic distance of 0.133. Furthermore, PVY-VN, PVY-YM, and PVY-MP were homologous with strain PVYC #M38377 at 94%, with a genetic distance of 0.138 (**Fig 5**).

Table 5. Nano-drop measurement of total RNA yield and impurities

PVY isolates	RNA yield (ng/µL)	Impurities Absorbance (260/280 nm ratio)
PVY-VN isolate	215.6	1.8
PVY-YM isolate	220.5	1.9
PVY--MP isolate	264.6	2.1

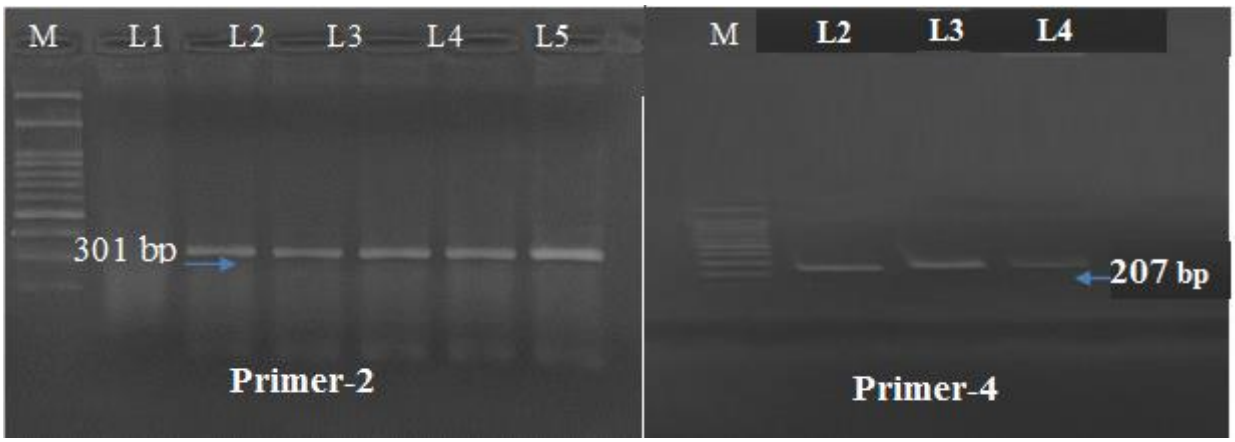

Fig 4. Photograph of agarose gel electrophoresis 1% showing RT-PCR amplicons of HC –silencing gene using RNA as a template of line L1 (PVY-VN), L3 (PVY-YM) and L4 (PVY-MP) isolates, L5 (Reference PVY isolate) and primer-2 & primer-4 showed expected size by primer-2 (301 bp) and primer-4 (207 bp) L1 (healthy negative control, M, 100 bp

Table 6. Screening efficacy design HC slicing primers for RT-PCR reaction with PVY-VN, PVY-YM, and PVY-MP isolates

PVY isolates	RT-PCR reaction using design HC silencing primer							
	primer- HC-1		primer- HC-2		primer- HC-3		primer- HC-4	
	Reaction	PCR product	Reaction	PCR product	Reaction	PCR product	Reaction	PCR product
PVY-VN	----	----	++++	301bp	-----	------	++	207bp
PVY-YM	----	----	++++	301bp	-----	------	++	207bp
PVY-MP	----	----	++++	301bp	-----	------	++	207bp

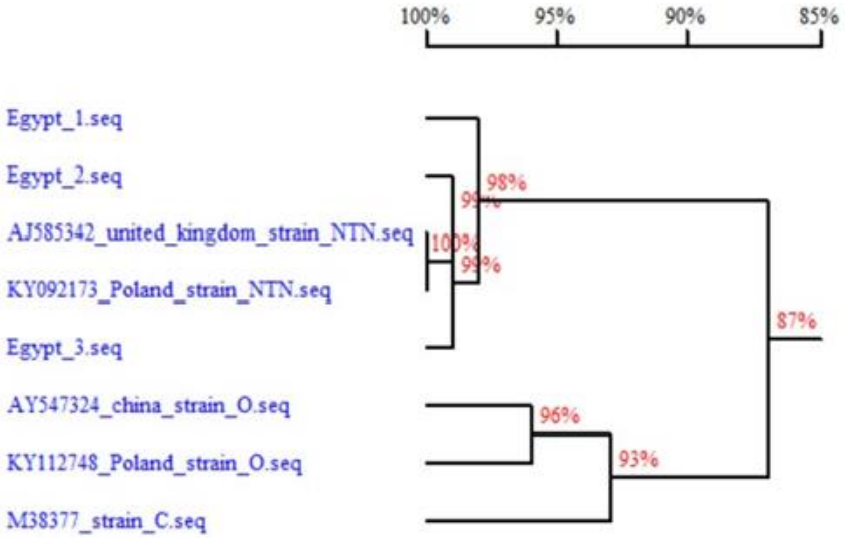

Fig 5. The phylogenetic tree represents the relationship between the three PVY isolates based on DNA sequence homology

Homology and Distance matrix representing the relationship between the three sequences of the HC-PRO RNA gene of PVY Egyptian isolates compared to five previously reported PVY isolates from NCBI, based on DNA sequence homology (Green et al 2017). The scale bar indicates the number of substitutions per nucleotide. A phylogenetic tree represented the relationship between the three PVY Egyptians and their comparison to previously reported PVY isolates based on DNA sequence homology of the HC gene (PVY) (**Fig 6)**.

3.4.4 HC-PRO silencing gene sequencing of PVY isolates

The DNA amplicons were returned as distinct fragments, as expected at 207 bp, for three PVY isolates with variable density. The sequencing of amplicons was performed using the PCR cycle sequencing reaction method. Primers were easily identified in either the forward or reverse direction in each sequence fragment and were readily used to piece together the individual sequences (Hu et al 2009). Sequences obtained for each primer for each isolate showed sufficient overlap and were used to form a single continuous sequence (contig). The three contigs were obtained by analyzing three sequence charts using the Finch TV program version 1.4 (Geospiza, USA), which displayed the partial nucleotide sequences of the HC silencing gene. The resulting sequences were compared with PVY species recorded on GenBank using the DNAMAN program, identifying PVY-1, PVY-2, and PVY-3 PVY isolates. Meanwhile, the alignment sequence analysis with PVY isolates in the gene bank indicated that VY-VN, PVY-YM, and PVY-MP isolates belonged to three sub-groups: PVYNTN, PVYN, and PVYo, with 90% sequence identity at the nucleotide level, respectively (Schmittgen and Livak 2008). The existence of PVY isolates that are genetically related but occur in geographically distinct areas, as noted in this work, suggests that they may be transmitted alongside infected plant materials between countries.

3.4.5 HC silencing gene sequence variability of PVY isolates

Sequence analysis based on multiple sequence alignment: The DNA sequences were subjected to variability analysis using DNAMAN version 7.2.0 (Wisconsin, Madison, USA). The three sequences of the HC silencing gene for PVY Egyptian isolates were aligned to identify the sequence registered in GenBank, as shown in **Fig 7**.

The phylogenetic relationships among the three isolates are illustrated in **Fig 8**. A multiple sequence alignment (MSA) was displayed, with the corresponding nucleotides occupying the same column. When a sequence lacks a corresponding residue due to a deletion event, the position is denoted by a dash, referred to as a gap. The alignment of multiple genes revealed conserved sites and the percentage of conservation at each position. Aligned residues share an evolutionary origin and exhibit sequence similarity.

3.4.6 Nucleotide sequence statistics

The replacement percentage of nitrogenous bases among the three isolates PVY-VN, PVY-YM & PVY-MP was 6.28% (similarity 93.72%). The similarity was high (94.2%) for PVY-VN with each of PVY-YM and PVY-MP, while it reached 98.5% between PVY-YM and PVY-MP related to 207 bp, as shown in **Table 7**. The three PVY isolates showed great similarities in the nucleotide sequences (99.67, 99.72 and 99.70 %)., respectively. For example: in the PVY-VN isolate, the nitrogen bases sites 5,42, 97,179, 188,197 198, 199, 201, 203, 204, 205, 207 were C, A, T, T, C, A, T, T, A, A, T, C, A but shifted to A, A, C, T, T, T, A, A, G, T, G, A, T in PVY-YM and to A, G, T, C, T, T, A, A, G, T, G, A, T in PVY-MP, respectively (**Table 8**).

The alignment of 207 bp of HC-PRO gene silencing nucleotide sequences among current isolates of PVY-VN, PVY-YM and PVY-MP revealed a replacement of nitrogenous bases. Sequence analysis indicated that variability could be derived from examining sequence statistics based on alignment information. The four types of nucleotides were counted for each of the three sequences individually. Subsequently, the G+C, A+T, and G+C/A+T ratios, along with the percentage of G+C, were determined. A distinct similarity was observed between PVY-VN and PVY-YM regarding adenine, cytosine, thymine, A+T, G+C, and the percentage of G+C, while PVY-3 displayed a more distant relationship, except for A+T and G+C/A+T. Notably, PVY-MP exhibited the highest content of adenine, guanine, G+C, the G+C/A+T ratio, and the percentage of C+G, as shown in **Table 9**.

The frequency of each nucleotide, G+C and A+T, was calculated for each sequence by dividing the number of each nucleotide by the total number of nucleotides, as shown in **Table 9.**

Distance matrix of 8 sequences

Egypt_1.seq	0
Egypt_2.seq	0.015 0
Egypt_3.seq	0.021 0.015 0
AJ585342_united_kingdom_strain_NTN.seq	0.021 0.005 0.010 0
KY092173_Poland_strain_NTN.seq	0.021 0.005 0.010 0.000 0
AY547324_china_strain_O.seq	0.133 0.138 0.133 0.144 0.144 0
KY112748_Poland_strain_O.seq	0.118 0.123 0.118 0.128 0.128 0.041 0
M38377_strain_C.seq	0.128 0.133 0.128 0.138 0.138 0.077 0.056 0

Homology matrix of 8 sequences

Egypt_1.seq	100%
Egypt_2.seq	98.5% 100%
Egypt_3.seq	97.9% 98.5% 100%
AJ585342_united_kingdom_strain_NTN.seq	97.9% 99.5% 99.0% 100%
KY092173_Poland_strain_NTN.seq	97.9% 99.5% 99.0% 100.0% 100%
AY547324_china_strain_O.seq	86.7% 86.2% 86.7% 85.6% 85.6% 100%
KY112748_Poland_strain_O.seq	88.2% 87.7% 88.2% 87.2% 87.2% 95.9% 100%
M38377_strain_C.seq	87.2% 86.7% 87.2% 86.2% 86.2% 92.3% 94.4% 100%

Fig 6. Distance and Homology matrix representing the relationship between the three sequences of the HC RNA gene of PVY Egyptian isolates comparison to five previously reported PVY isolates from NCBI based on DNA sequence homology

Fig 7. Nucleotides equence alignment of HC silencing gene sequence among PVY isolates

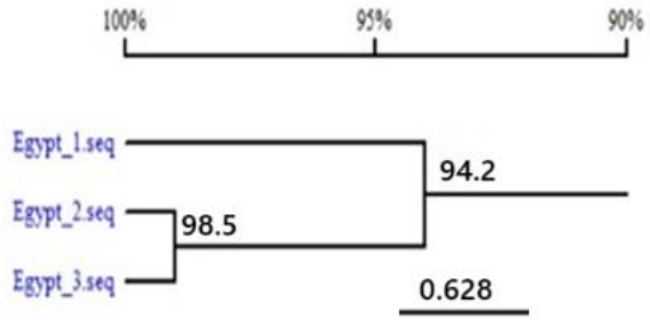

Fig 8. Phylogenetic tree representing the relationship between the three PVY Egyptian isolates based on DNA sequence homology

Table 7. Homology and genetic Distance matrix representing the relationship between the three sequences of the HC RNA gene of PVY current isolates

% of similarity			
PVY isolates	**PVY-VN**	**PVY-YM**	**PVY-MP**
PVY-VN	0	94.2	94.2
PVY-YM	0.579	0	98.5
PVY-MP	o.579	0.150	0
Genetic distance			

Table 8. Replacement situation of nitrogen base in nucleotide sequences of HC- gene for three PVY isolates

Current isolates	**Situation replacement of nitrogenous bases**												
	5	42	97	179	188	197	198	199	201	203	204	205	207
PVY-VN	C	A	T	T	C	A	T	T	A	A	T	C	A
PVY-YM	A	A	C	T	T	T	A	A	G	T	G	A	T
PVY-MP	A	G	T	C	T	T	A	A	G	T	G	A	T

Table 9. Count of nucleotides individually, in combined form, G+C/A+T and %G+C for the three PVY isolates

Nucleotide	**PVY isolates (nd =208)**		
	PVY-VN	**PVY-YM**	**PVY-MP**
Adenine (A)	53	55	55
Cytosine (C)	43	43	42
Guanine (G)	40	39	40
Thymine (T)	59	58	58
G+C	83	82	82
% G+C	42.56%	42.05%	42.05%
A+T	112	113	113
% A+T	57.57%	57.94%	57.94%
G+C/A+T	0.74	0.72	0.72

Sequences of the most variable regions of specific genes are increasingly valuable for identifying closely related species in the current study. The results of BLAST sequencing and the phylogenetic tree of the HC gene were sufficient for species delimitation of the PVY isolates. It was reported that analysis based on HC-gene sequences is ineffective in distinguishing closely related species within the Potyvirus genus. The CP gene sequences are highly informative and aid in investigating closely related strains at the species level (Elwan et al 2021).

Different partial nucleotide sequences of the HC silencing gene were identified as PVY-VN, PVY-YM, and PVY-MP isolates (207 bp). These results are relatively consistent with those obtained by Schubert et al (2014), who isolated three PVY isolates from infected potato plants in fields across various governorates in Egypt and identified them using the HC silencing gene. They found three partial sequences measuring 207 bp and designated them as Egypt-1.seq, Egypt-2.seq, and Egypt-3.seq. Phylogenetic tree and molecular evolutionary analyses were conducted using DNAMAN. Based on multiple sequence analysis (MSA) of the three PVY isolates and PVY isolates recorded in GenBank, variability of the HC-PRO-silencing gene sequence was evaluated, and sequence alignment showed homology among bases attributed to base pair substitutions.

It was noted that the PVY-VN, PVY-YM, and PVY-MP isolates from naturally infected potato plants are very similar but so far from potato fields in different governorates in Egypt (Elwan et al 2021). This close genetic similarity suggests that such climate changes have little effect on the genetic constitution of the three isolates, whilst the adverse events in the summer season caused genetic rearrangement of their genome. Sequential variability is common and can be attributed to a strong mutagenic character that is influenced by climate change and the chemical and biological composition of plants.

Table 10. PVY accumulation Level related to HC-PRO slicing gene expression in potato plants infected with three PVY isolatesunder greenhouse conditions

PVY Accumulation / PVY isolates	Level PVY Accumulation (CT value)			
	CP gene	Actin	ΔΔCT	RQ
Mock	Und.	25.0	Und.	Und.
PVY strain	35.32-fold c	22.5	− 9.5	18.5
Venial necrosis(PVY-VN)	28.75-fold a	23.4	− 6.5	15.8
Yellow mosaic (PVY-YM)	30.45-fold b	26.2	− 8.7	14.6
Mottling pattern(PVY-MP)	32.85-fold c	25.4	− 5.6	10.5
The average values of the columns with the same letter do not differ significantly. at P value > 0.05				

Where, Cox = Reference gene, Fold Change (RQ) = 2-ΔΔCT
ΔΔCt = Δ CT (test sample) − Δ CT (calibrator sample)
Δ CT (test sample) = CT (target of interest) − CT (reference gene in sample) and
Δ CT (calibrator sample) = CT (target in control) − CT (reference gene in control).

3.5 PVY accumulation Level in infected potato plants with three PVY isolates

The levels of PVY accumulation were determined by qRT-PCR related to the HC-PRO silencing gene in infected potato plants. The PVY accumulation levels in infected potato plants with the PVY-YN, PVY-YM, and PVY-MP isolates show significant variability compared to the PVY reference strain (Schmittgen and Livak 2008). PVY accumulation was quantified based on the cycle threshold (Ct) value ratio of the PVY-HC-PRO silencing gene to the potato internal control actin gene. The qRT-PCR data, presented in **Table 10**, indicated that the accumulation levels of PVY-CP were 28.75, 30.46, and 32.82-fold higher in the infected potato plants (PVY-YN, PVY-YM, and PVY-MP, respectively) compared to the PVY reference strain (35.32-fold). No PVY was detected in the mock potato plants.

4 Conclusion

All the obtained data in the present study revealed the diversity of potato Y virus isolates from different points of view with regard to the evolutionary and taxonomic impact in addition to the behaviors of the PVY-VN, PVY-YM and PVY-MP isolates in the potato plant and the adaptation of the three isolates to the environmental conditions that affect both the plant and the virus as a result of the climate changes. The study of the biological, serological and molecular characteristics showed the distinctive behavior of the isolates (PVY-VN, PVY-YM and PVY-MP), especially the molecular characteristics that indicate that they can be considered strains of the potato Y virus genus.

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