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Nucleotide Sequence for Two Genes of *Egyptian Barley Yellow Dwarf Virus* - PAV Isolate and their Relationships to other Luteovirus

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Abstract

Barley Yellow Dwarf Virus (BYDV- PAV) isolated from *Wheat* plants grown in Egypt has been characterized. Two coding regions of the *Barley yellow dwarf virus* (BYDV- PAV) isolate for the polymerase gene (P1) located in open reading frame (ORF1) and coat protein gene (CP) located in open reading frame (ORF3) were targeted. Two sets of specific primerswere designed according to of BYDV-PAV isolate in Genbank. The DNA fragments of ORF1and ORF3 of an Egyptian isolate of BYDV- PAV were cloned and sequenced. The sequence contained a full-length ORF1 coding for the viral polymerase gene (P1). It comprises 910 ntin length encodes a predicted polypeptide chain of 303 amino acids with a M(r) of 34.67. On the other hand, the sequence data for the ORF3 coding for the viral coat protein revealed that it was 603 bp in length encodes a predicted protein 200 amino acids, with molecular weight of 21.96 KDa. However, the phylogenetic homology tree based on the multiple sequence alignments of the Egyptian isolate Egy-Wz with those isolates available in NCBI GenBank revealed that the polymerase gene (P1) shared 76.5 %-99 % and 71.6% - 93.2% sequence identities at amino acid and nucleotide levels with the 05GG2, PAV 014 and PAV014, PAV-Aus isolates respectively. On the other hand, the coat protein gene (CP) showed 85.1% -99.5% and 89.7%-99.2% sequencesimilarity with two isolates 06KM14 and 05GG2 at the amino acid and nucleotide level, respectively.

Keywords: RT-PCR;ORF; Sequencing;Cloning; *Barley yellow dwarf virus*; BYDV-PAV isolate.

Introduction

Barley yellow dwarf virus one of the most important viral diseases. It has a wide host range in the family Gramineae including important economic cereal crops wheat, barley, maize, rice, sorghum, oats, rye, triticale, and many of weeds [1]. Symptoms are different according to the host and cultivar, age of the host at the time of infection, physiological conditions, virus isolate, and the environment [2]. It has become an important reason for cereal production limitation in all of the major world sources of grain. Taxonomically, the viruses responsible for BYD disease belong to the family Luteoviridae: genus Luteovirus, BYDV-PAV, BYDV-MAV, BYDV- PAS [3]. The most damaging isolate of *barley yellow dwarf virus*(BYDV) is PAV isolate [4]. It causes serious losses all over the world in wheat (17%), barley (15%) [1]. BYDV is considered as a complex biological system, in spite of that in recent yearsfurthers studies have been done and revealed significant information which facilitate the understanding of the molecular biology of the *barley yellow dwarf viruses* (BYDV). BYDV has a positive sense*ssRNA*genome of approximately 5.6 kb that has encodes six open reading frames (ORFs), and produce three sub genomic RNAs [5]. Both ORF1 and ORF2 are the only essential for replication of the virus in plant [6]. The coat protein (CP) is coded by ORF3, while ORF4, which is sub genomic RNA, translated within the sequence that codes for ORF3 in different reading frame that code for a protein necessary for plantsinfection.

`Lines that have natural resistance for BYDV or tolerant at a significant level by the breeders are limited. Miller et al. [7] have completed the entire genome nucleotide sequence of barley yellow dwarf PAV serotype (BYDV-PAV) in 1988. Thegenome consists of 5677 nucleotides, which encodes six open reading frames (6 ORFs) [7]. After that, number of other luteoviruses was determined successfully.This provides better understanding of BYDV molecular mechanisms in order to help for controlling the disease [8]. Genetic engineering has provided a powerful tool for virus resistance [9]. Genetic resistance is safe for the environment and with reasonable cost; however BYDV natural resistance genes are few [10,11]. Only few natural resistance genes for wheat, oat, rice, maize and barley, which are, consider the most economic important hosts are available. A method of the advantages of genetic engineering is that the resistant genes which is not existed in nature can be constructed, in order that resistance by genetically engineered is very important method and useful for BYDVs diseases [12]. Besides the problems of conventional plant, breeding can be avoided by genetic engineering by introducing single resistance genes into commercial cultivars without carrying any linked deleterious genes [12]. Transgenic plants have been obtained by introducing different artificial resistance genes [13 – 15]. The mission of the artificial resistance gene is disturbing and interfering the viral replication cycle, and host-viral interaction [16]. The viral coat protein is the first approach, which used very widely to be introduced to the host plant genome [17]. In addition, the viral replicas genes are used for artificial resistance. Transgenic plants expressing RNA-dependent RNA polymerase gene for tobacco mosaic virus (TMV) were immune to be infected by TMV [18].

Here, we report the sequence of Two coding regions of the *Barley yellow dwarf virus* (BYDV) PAV isolate for the polymerase gene located in open reading frame (ORF1) and coat protein gene located in open reading frame (ORF3) and study the genetic diversity found in BYDV PAV compared with those of other luteoviruses.

Material and Methods

Virus Source

Barley yellow dwarf virus (BYDV) PAV was isolated previously from naturally infected wheat plants (*Triticumspp*) collected from Giza Governorate and identified based on the viral symptoms, and serological detection.

Total RNA extraction

Total RNA was extracted from purified virus particles of BYDV-PAV isolate, and isolated with an equal volume of buffered *phenol*: *chloroform*: *Isoamyl alcohol* (25:24:1, v/v/v) according to [19].

Primer Design

For the PCR reaction, several primers were designed using two of the sequences of BYDV-PAV available in GenBank. Two coding regions in the BYDV-PAV genome were targeted, the polymerase located in open reading frame (ORF1), and the coat protein (CP) located in ORF3 [20].

For (ORF1) polymerase gene (P1), the primers were designed according to unpublished data [8]BEgy3 (F), BEgy4(R). For (ORF3) coat protein gene (CP) two primers were designed according to Miller et al. [8], BEgy1(F), and BEgy2(R),with restriction site for Bam H1 at the terminal 5` and aa tail (Table1).

Polymerase gene P1 (ORF1)

RT reaction was carried out in 25µl reaction volume containing 5 µl of RNA template, 5 µl BEgy4 (R) reverse primer in concentration 20 pmol/ µl, and DEPC treated water was added to volume 12.5 µl. The tubes were heated to 70˚C for 5 min,and then cooled immediately on ice, and the tubeswere spint briefly to collect the solution at the bottom of the tube. The following components were added in the following order, 5 µl M-MLV, 5x reaction buffer, 5 µl of dNTPs 2.5 mM ,0.6 µl of rRNasin, 40u/ µl (Promega Cat # N211A),1 µl of M-MLV RT 200u/µl (PromegaCat# M1701),Nuclease –Free water to volume

25µl. The tubes were mixed gently by flicking, and

PCR reaction

The PCR reaction mixture using specific BYDV-P1 primers was prepared by using 2 l of the resulting cDNA. The BYDV cDNA was transferred to a tube containing 10 l of 1X polymerase chain reaction buffer (10mM Tris-HCl, pH 8.3, 50 mMKCl, 0.01% gelatin (TaKaRa code# DRR100A)), 2.5 mM MgCl2, 200M dNTPs, 10 pmol of BEgy3(F) and BEgy4(R) of forward and reverse primer respectively, 1µ of Taq DNA polymerase ((Ex Taq Takara)). The amplification proceeded in thethermo cycler (Uno II Biometra) at 94°C for 2 min, and through 40 cycles of 94°C for 30 s and 52°C for 30s and 72°C for 30 s, with a final step at 72°C for 10 min. Products were electrophoresed in 1% agarose gel in TAE (40mMTris–acetate, 1mM EDTA, pH 8.0) and stained with ethidium bromide.

Coat Protein Gene CP (ORF3)

One step RT-PCR

Promega kit Access RT-PCR introductory system (cat# A1260) was used for RT-PCR in total volume of 50 µl.For one reaction 10 µl of AMV/ Tfl 5x reaction buffer was added in ependorf tube, then 10 pmol, of each downstream BEgy2 –R and upstream primer BEgy1-F were add. 200M of dNTPs Mix was used. 2 µl of 25mM MgSo4 and 29 µl of nuclease free water were added, then the contents were mixed well using pipette, one microliter of the AMV reverse transcriptase and Tf l DNA polymerase were added, and gently vortexed. RNA template was finally added for total volume 50 µl.

The program file used as follow: one cycle at 48°C for 45 min for cDNA synthesizes. For DNA amplification, 1cycle at 94°C for 2 min, and 45 cycles were done at 94°C for 30 sec, 55°C for 1 min, and 68°C for 2 min was used followed by one cycle at 68°C for 7 min for final extension. PCR was done using Perkin Elmer Gen amp PCR system 2400. Products were electrophoresed in 1.2% agarose gel in TAE, and stained with ethidium bromide.

PCR Product Purification

PCR product was purified using Shanghai Shenyou Biotechnology Co., LTd Kit # K0901(100).

Cloning into pGEM-T Easy

The BYDV-PAVPCR amplified product was directly cloned into linearized and thymidylated pGEM -T-easy vector according Promega kit cat#A1360. The incubated for 60 min at 42˚C.

constructswere transformed into *E. coli* competent cells according to Sambrooket al. [21]. Blue/white colonies had screened up to select the bacterial colonies transformed with recombinant pGEM plasmids using the PCR technique. Clones from transformed cells were purified using the Wizard minipreps DNA purification system (Promega Corporation MD) and sequenced directly.

Sequence and Phylogenetic Analysis

The recombinants of BYDV-PAV-ORF3 and BYDV-PAV-ORF1 were obtained and submitted for sequencing which was carried out in both the forward and reverse directions with M13 primer, using an (ABI PRIM model 377) sequencer.Nucleotide sequence data were compiled and analyzed.Database searches were performed with Basic Local Alignment Search Tool (BLAST) program at National Center for Biotechnology Information (NCBI), National Institute of Health. Multiple sequence alignments for nucleotide and deduced amino acids sequences were obtained using CLUSTAL W program, and the phylogenetic tree was constructed using Geneious Pro 8.0.5sofware.

Primer design specific for the Egyptian isolate (BYDV-PAV)

The clone that found to be presented cDNA for the ORF1 and ORF3genes, were re-amplified using several new primers, which were designed based on the sequences of the Egyptian isolate BYDV-PAV- Egy-Wz, with restriction site and poly A tail as shown in Table (2). The set of primers used for (ORF3) were BEgy-Wz1(F) and BEgy-Wz2(F), and for the (ORF1) were BEgy-Wz3(F) BEgy and Wz4(R).

PCR Reaction

The PCR reaction has been done using the Premix TaqTaKaRa [(Ex TaqTM version) code 3#DRR 003A] for both genes in total volume 50 µl. The set of primers used for the coat protein gene CP (ORF3) were BEgy-Wz1(F) and BEgy-Wz2(R), and for the polymerase gene P1(ORF1) were BEgy-Wz3(F) BEgy-Wz4(R).

The program was used for amplification of ORF1 was as follow: 94˚C for 5 min followed by 45cycle 94˚C for 30 s, 54˚C for 30 s and 72˚C for 1min, and a final incubation at 72˚C for 7 min. The amplification program for of ORF3 was as follow: 94˚C for 2 min followed by 35cycle 94˚C for 30 s, 55˚C for 30 s and 72˚C for 1min, and a final incubation at 72˚C for 7 min. The amplified products were electrophoresed in 1% agarose gels in TAE and stained with ethidium bromide.

Sub-cloning and Sequencing

The resulting PCR fragments for ORF1and ORF3 were purified using Shanghai shenyou Biotechnology Co., LTd Kit # K0901 (100) as was mentioned previously. The PCR product of ORF1 hasKpnI and Sac1 restriction sites, and the product of ORF3 have positions for XhoI and SacI restriction enzymes. The cDNA products were cloned into polylinker region of bacterial expression vector (pEmue-mes-N). The recombinants of BYDV-PAV-Egy-Wzfor ORF1and ORF3 were obtained and submitted for sequencing for each region to confirm the presence of the unmodified cDNA using an (ABI PRIM model 377) sequencer.Nucleotide sequence data were compiled and analyzed.

Results

RT-PCR and One step PCR

Total RNA of purified virus was extracted from wheat tissue infected with BYDV-PAV Egyptian isolate. Two set of primers were designed according to isolates of BYDV-PAV available in the GenBank. A cDNA fragment of ~1kb were amplified by RT-PCR reaction with primers BEgy3(F) and BEgy4 (R) designed for amplifying the P1 gene (Figure 1), and a cDNA fragment of ~600 bps were amplified by PCR reaction with primer BEgy1(F) and BEgy2(R) designed for amplifying the CP gene encoding region of BYDV-PAV isolate(Figure 2).

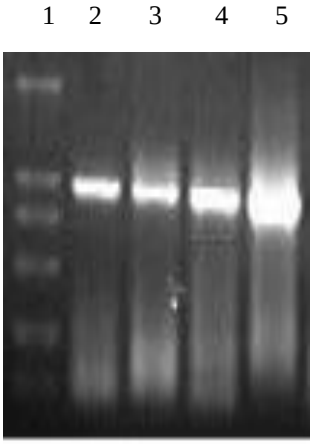


Figure 1: Agarose gel electrophoretic analysis of RT-PCR amplification products for the P1 gene obtained with primers BEg3 & BEg4. Lane 1: DNA Marker (M.W. 2 kb), from lane 2 to 5 RT- PCR from partially purified total RNA preparation.

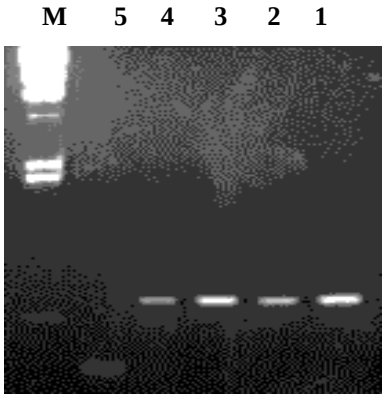


Figure 2: Agarose gel electrophoresis analysis of reverse transcription polymerase chain reaction (RT –PCR) amplified products for the CP gene obtained with primers BEgy1 (F),and BEgy2(R). Lane Mr: DNA Marker (M.W.15 kb), lane 1 the kit positive control, lane 2 to 4 from total RNA preparation of partial purified of wheat leaves infected with BYDV –PAV isolate, lane 5 from the purified particles.

Table 1: Primer sequences designed from isolates of BYDV-PAVin the GeneBank.

Primer	Sequence	Site	ACC.NO.	Product size	Amplified region
BEgy1 (F)	AA GGAT CC C ACT AGA GAG GTG GTG AAT G	2841-2861	X07653	~600 bp	ORF3
BEgy2 (R)	AA GGAT CC T CCG GTG TTG AGG AGT CTA CCT A	3458-3481			
BEgy3 (F)	AA GGAT CC GTC TGA CAC CGT ACT GCC CC Bam H1	100-120	NC002160	~1kb	ORF1
BEgy4 (R)	AA GGAT CC CAA AAC CGG AGG CGG CAC AG	1170-1190			

Table 2: Primer sequences designed according to the Egyptian isolate (BYDV-PAV) sequence for polymerase and coat protein gene.

Primer	Primer Sequence in `5- `3 orientation	Product size	Amplified region
BEgy-Wz1(F) Sense	aa**CTCGAG**ATGAATTCAGTAGGCCGTAG **Xhox**	~600	ORF3
BEgy-Wz2(R) antisense	aa**GAGCTC** CTA TT T GGC CGT CAT CAAAC **SacI**		
BEgy-Wz3(F) Sense	aa**GGTACC** ATGTTTTTCGAAATACTTAT **KpnI**	~1kb	ORF1
BEgy-Wz4(R) Antisense	aa**GAGCTC**ACATCGATGCGACTTGCTTT **SacI**		

Table 3: The barley yellow dwarf virus isolatesavailable in NCBI Gene Bank with their Accession numbers, their Country of origin and their host plant.

Isolate name	Country of origin	ACC. No.	Host plant
05GG2	China	EU332309	Wheat
06KM14	China	EU332332	Wheat
FL3	France	AT223587	Ryegrass
PAV-AUS	Australia	M21347	Barley
FH3	France	AJ223589	Barley
MA9513	Morocco	AJ007924	Barley
P-PAV	United states	X17261	Oat
PAS-129	United states	DQ285682	Oat
FL2	France	AJ223586	Ryegrass
FH1	France	AJ223588	Barley
MA9514	Morocco	AJ007925	
MA9516	Morocco	AJ007926	

Table 4: % Identity of nucleotide alignment of the Egyptian isolate BYDV-PAV Egy- Wz of the polymerase gene (P1)with other isolates of BYDV-PAV available in GenBank by using Genious pro 8-0-5 Software.

	05GG2	05ZZ7	Egy-Wz	MAV	PAS-129	PAV 014	PAV-Ast	PAV-III	RPV 046
05GG2		70.620%	92.896%	93.005%	75.027%	93.989%	93.115%	93.231%	4.119%
05ZZ7	70.620%		71.616%	69.760%	76.009%	70.415%	71.397%	70.697%	3.215%
Egy-Wz	92.896%	71.616%		91.868%	76.974%	93.297%	93.297%	92.215%	4.233%
MAV	93.005%	69.760%	91.868%		75.329%	96.374%	92.418%	95.614%	4.119%
PAS-129	75.027%	76.009%	76.974%	75.329%		76.206%	76.316%	77.218%	3.330%
PAV 014	93.989%	70.415%	93.297%	96.374%	76.206%		93.516%	96.491%	4.005%
PAV-Ast	93.115%	71.397%	93.297%	92.418%	76.316%	93.516%		92.544%	4.348%
PAV-III	93.231%	70.697%	92.215%	95.614%	77.218%	96.491%	92.544%		4.233%
RPV 046	4.119%	3.215%	4.233%	4.119%	3.330%	4.005%	4.348%	4.233%	

Table 5: % Identity of amino acids alignment of the Egyptian isolate BYDV-PAV Egy- Wz of the polymerase gene (P1)with other isolates of BYDV-PAV available in GenBank by using Genious pro 8-0-5 Software.

	05GG2	05ZZ7	Egy-Wz	MAV	PAS-129	PAV 014	PAV-Aus	PAV-III	RPV 046
05GG2		75.828%	99.010%	97.360%	78.808%	98.680%	98.020%	98.020%	26.667%
05ZZ7	75.828%		76.490%	76.490%	82.508%	76.490%	76.490%	77.483%	12.500%
Egy-Wz	99.010%	76.490%		97.690%	79.139%	99.010%	98.350%	98.350%	26.667%
MAV	97.360%	76.490%	97.690%		79.139%	98.020%	97.360%	98.680%	26.667%
PAS-129	78.808%	82.508%	79.139%	79.139%		79.470%	79.470%	79.801%	12.500%
PAV 014	98.680%	76.490%	99.010%	98.020%	79.470%		98.680%	98.680%	26.667%
PAV-Aus	98.020%	76.490%	98.350%	97.360%	79.470%	98.680%		98.020%	26.667%
PAV-III	98.020%	77.483%	98.350%	98.680%	79.801%	98.680%	98.020%		26.667%
RPV 046	26.667%	12.500%	26.667%	26.667%	12.500%	26.667%	26.667%	26.667%	

Table 6: Nucleotide sequence percentage identity of the coat protein gene for the BYDV-PAV Egyptian isolate Egy-Wz and other isolates of BYDV-PAV available in GenBank W using Genious pro 8-0-5 Software.

	MA9516	MA9514	FL2	FH1	PAS-129	P-PAV	FH3	MA9513	06KM14	05GG2	Egy-Wz	FL3	PAV-Aus	MAV
MA9516		98.839%	98.176%	98.673%	95.357%	89.221%	88.226%	91.045%	88.723%	89.055%	88.723%	88.889%	89.055%	75.743%
MA9514	98.839%		97.678%	98.176%	94.859%	89.718%	88.557%	89.884%	89.221%	89.552%	89.221%	89.386%	89.552%	76.073%
FL2	98.176%	97.678%		99.171%	96.186%	90.216%	88.889%	90.050%	89.718%	89.718%	89.718%	89.552%	89.552%	75.743%
FH1	98.673%	98.176%	99.171%		96.352%	90.050%	88.889%	89.884%	89.552%	89.884%	89.552%	89.221%	89.552%	75.908%
PAS-129	95.357%	94.859%	96.186%	96.352%		89.884%	88.557%	89.552%	89.884%	90.216%	89.884%	89.884%	89.386%	76.073%
P-PAV	89.221%	89.718%	90.216%	90.050%	89.884%		97.844%	97.678%	96.186%	96.186%	96.683%	96.517%	95.025%	77.063%
FH3	88.226%	88.557%	88.889%	88.889%	88.557%	97.844%		96.352%	94.859%	94.859%	95.025%	94.859%	93.698%	76.073%
MA9513	91.045%	89.884%	90.050%	89.884%	89.552%	97.678%	96.352%		94.859%	94.859%	95.025%	94.859%	93.367%	76.238%
06KM14	88.723%	89.221%	89.718%	89.552%	89.884%	96.186%	94.859%	94.859%		99.337%	99.171%	98.342%	97.512%	77.393%
05GG2	89.055%	89.552%	89.718%	89.884%	90.216%	96.186%	94.859%	94.859%	99.337%		99.171%	98.342%	97.512%	77.063%
Egy-Wz	88.723%	89.221%	89.718%	89.552%	89.884%	96.683%	95.025%	95.025%	99.171%	99.171%		98.839%	97.678%	77.228%
FL3	88.889%	89.386%	89.552%	89.221%	89.884%	96.517%	94.859%	94.859%	98.342%	98.342%	98.839%		97.512%	76.403%
PAV-Aus	89.055%	89.552%	89.552%	89.552%	89.386%	95.025%	93.698%	93.367%	97.512%	97.512%	97.678%	97.512%		76.568%
MAV	75.743%	76.073%	75.743%	75.908%	76.073%	77.063%	76.073%	76.238%	77.393%	77.063%	77.228%	76.403%	76.568%	

Table 7: The similarity percentage for coat protein Amino acid sequence of the BYDV-PAV Egyptian isolate Egy-Wz and other isolates of BYDV-PAV available in GenBank .by using Genious pro 8-0-5 Software.

	05GG2	06KM14	Egy-Wz	FH1	FH3	FL2	FL3	MA9513	MA9514	MA9516	MAV	PAS-129	PAV-Aus	P-PAV
05GG2		100%	99.500%	86.000%	95.500%	86.500%	98.500%	96.500%	85.500%	85.000%	71.642%	87.000%	98.000%	98.000%
06KM14	100%		99.500%	86.000%	95.500%	86.500%	98.500%	96.500%	85.500%	85.000%	71.642%	87.000%	98.000%	98.000%
Egy-Wz	99.500%	99.500%		85.500%	95.000%	86.000%	98.000%	96.000%	85.000%	84.500%	72.139%	86.500%	97.500%	97.500%
FH1	86.000%	86.000%	85.500%		82.500%	99.500%	85.000%	84.500%	96.500%	97.000%	69.154%	95.500%	85.000%	85.000%
FH3	95.500%	95.500%	95.000%	82.500%		83.000%	95.000%	94.000%	82.000%	82.000%	69.154%	83.500%	94.500%	96.000%
FL2	86.500%	86.500%	86.000%	99.500%	83.000%		85.500%	85.000%	97.000%	97.500%	69.652%	96.000%	85.500%	85.500%
FL3	98.500%	98.500%	98.000%	85.000%	95.000%	85.500%		95.000%	84.500%	84.000%	70.149%	86.000%	97.500%	97.500%
MA9513	96.500%	96.500%	96.000%	84.500%	94.000%	85.000%	95.000%		86.000%	87.500%	70.647%	85.500%	94.500%	96.500%
MA9514	85.500%	85.500%	85.000%	96.500%	82.000%	97.000%	84.500%	86.000%		98.500%	69.154%	93.000%	84.500%	84.500%
MA9516	85.000%	85.000%	84.500%	97.000%	82.000%	97.500%	84.000%	87.500%	98.500%		69.652%	93.500%	84.000%	84.000%
MAV	71.642%	71.642%	72.139%	69.154%	69.154%	69.652%	70.149%	70.647%	69.154%	69.652%		69.652%	71.642%	71.144%
PAS-129	87.000%	87.000%	86.500%	95.500%	83.500%	96.000%	86.000%	85.500%	93.000%	93.500%	69.652%		86.500%	86.000%
PAV-Aus	98.000%	98.000%	97.500%	85.000%	94.500%	85.500%	97.500%	94.500%	84.500%	84.000%	71.642%	86.500%		97.000%
P-PAV	98.000%	98.000%	97.500%	85.000%	96.000%	85.500%	97.500%	96.500%	84.500%	84.000%	71.144%	86.000%	97.000%	

Cloning and Transformation

The amplified PCR product of both genes (polymerase gene P1 and the coat protein gene) were ligated to pGEM-T easy vector and transformed into *E. coli* competent cells. The DNA was prepared from the recombinant plasmids and the insert was sequenced.

Sequence analysis

The PCR product for the coat protein (CP) was 603 bp in length encodes for 200 amino acids, with molecular weight of 21.96 KDa, while the polymerase gene (P1) was 910 bp in length and encodes a predicted protein of 303 deduced amino acids with molecular weight 34.67 KDa. The nucleotide sequences of the coat protein(CP) and polymerase gene (P1) of the Egyptian isolate BYDV-PAV have been deposited into the Gene Bank withAcc.No. KM046987-KM04988, respectively.Multiple sequence alignments of nucleotide and deduced amino acids sequences were obtained with CLUSTAL W and Geneious pro 8.0.5 software.

The multiple sequence alignment of the obtained genes of BYDV-PAV for the Egyptian isolate Egy-Wz with those isolates available in NCBI Gene Bank with their Accession numbers (Tables 3&4) revealed that the polymerase gene (P1) shared 93.2% with the PAV014 and PAV-Aus sequence identities at nucleotide level. In the meantime, it showed the lowest nucleotide identities 71.6% with 05ZZ7 isolate (Table 4).In Addition, it shared 99.0% with 05GG2 and PAV 014 sequence identities at amino acid levels, while it showed the lowest identity 76.5% with the 05ZZ7 isolate (Table 5).

The coat protein gene of the Egyptian Isolate Egy-Wz shared 99.2% sequence identities with the two isolates 06KM14 and 05GG2 at nucleotide level. The CP of Egyptian isolate was far from MA9516 isolate (88.7% similarity) as shown in Table 6. On the other hand, it shared 99.5% with the two isolates 06KM14 and 05GG2 sequence identities at amino acid level, while it showed the lowest identity 85.1% with the MA9516, the England and Australian isolates (Table 7).

The Egyptian isolate coat protein sequence was examined for the group A and group B serotype. The nucleotide sequence identity of the coat protein ranged from

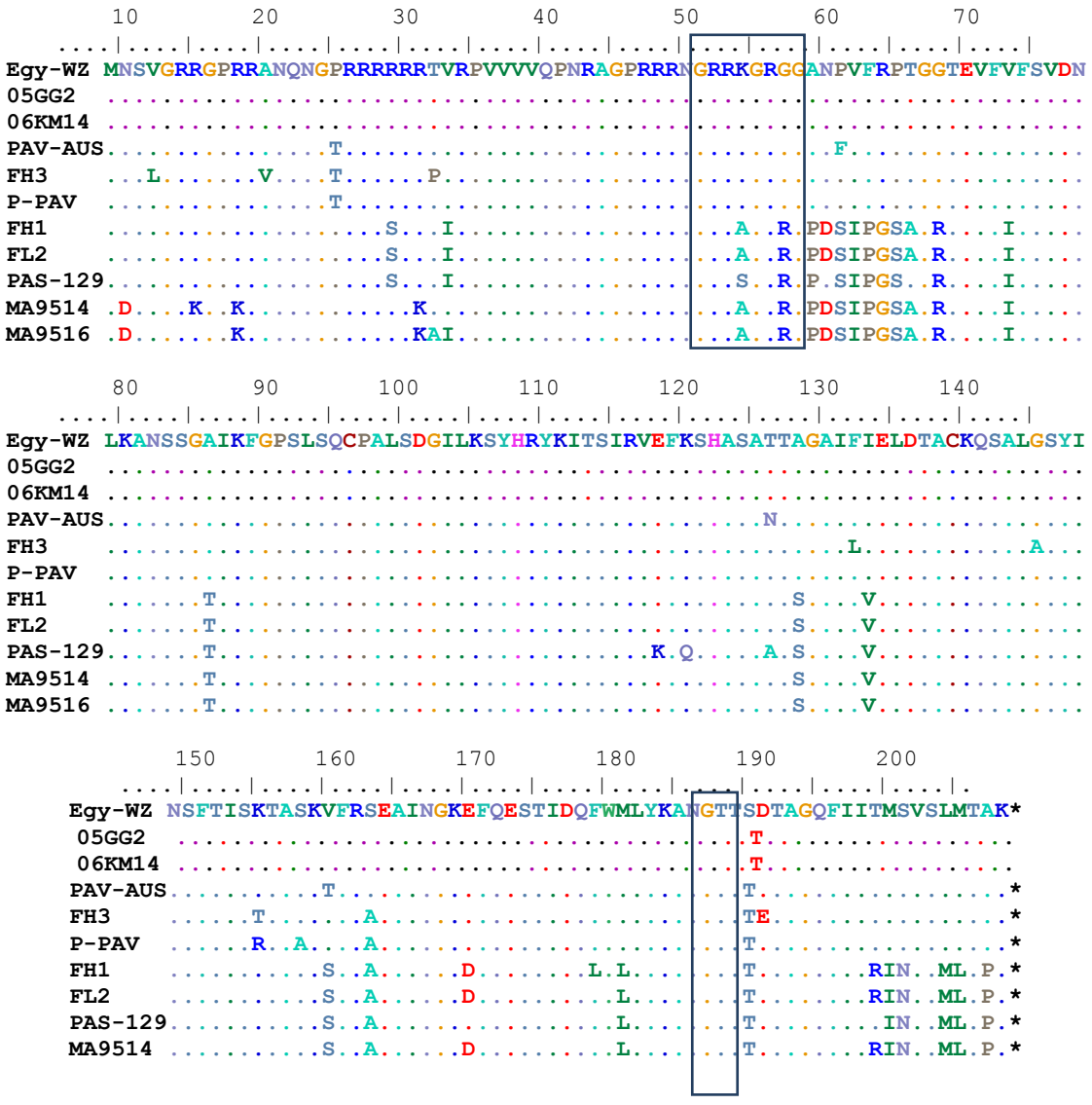
95 to 99 in-group A and from 88 to 89.8 in-group B (Table 6).Coincidentally, the Amino acid identity of the capsid proteins ranged from 96 to 99.5% in-group A and from 84 to 86% in-group B (Table 7). The most nucleotide variable region was located at position 136 to 172 and 513 to 595. While the main amino acid differences were located at, amino acid positions 51 to 58 and at the position 191 to 193 of the C – terminal region. On the other hand, the most constant region located at nucleotide position 74 to 135 (Figure 3).

Phylogenetic Relationships between Isolates

The phylogenetic tree was constructed using Geneious pro 8.0.5 software to determine the phylogenetic relationships between the Egyptian isolate Egy-Wz and other PAV and PAS isolates available in Gene bank. In the phylogenetic tree for the polymerase gene P1 the sequence identity for the nucleotides of sequence of the Egy-Wz isolate demonstrated that the Egyptian isolate located in a cluster with PAV-Aus and 05GG2 as shown in Figure 4. While the phylogenetic tree for the amino acids indicate that, the Egyptian isolate located at the same cluster with the same isolates including PAV 014 (data not shown).The sequence identity for the nucleotides and the amino acids of the coat protein for the Egy-Wz isolate demonstrated that the Egy-Wz located in the same cluster with the other PAV isolates in group A as shown in Figures 5&6.

The PCR with Specific Primers

The resulting PCR fragments for ORF3 (CP coding region) was at the expected size about 603 bp, and for ORF 1 (P1 gene) it was about 910 bp. The PCR products were cloned into polylinker region of bacterial expression vector (pEmue- mes-N), and sequenced to confirm the sequence for both ORF1 and ORF3 of the Egyptian isolate BYDV-PAV-Egy-Wz.



MA9516 .....S..A.....D.....L.....T.....RIN..ML.P.*

Figure 3: Multiple alignment of deduced amino acid sequence analysis of coat proteins of different barley yellow dwarf virus serotype (groupA &B) isolates available in Genbankand the Egyptian isolate Egy-Wz- PAV. The most Amino acids variable regions are boxed.

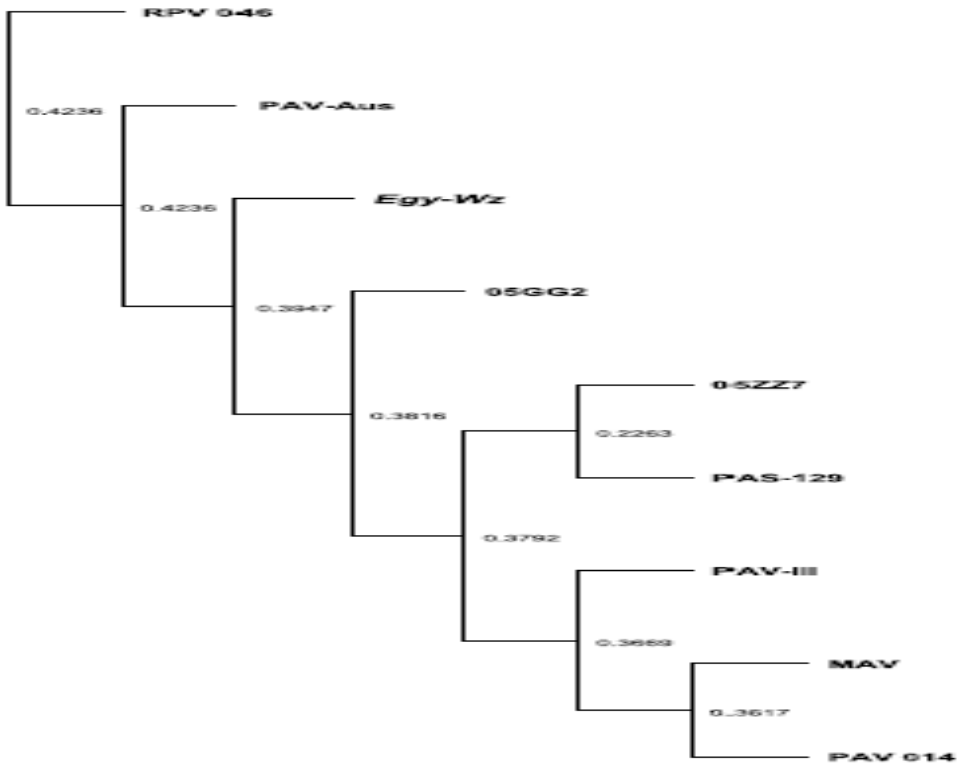


Figure4: Phylogenetic relationship of the BYDV-PAV of the Egyptian isolate (Egy-Wz) based on multiple nucleotide sequence alignments of the Polymerase gene (P1) and other BYDV-PAV isolates available in GenBank, instructed by CLUSTAL W using Genious pro 8-0-5 Software.

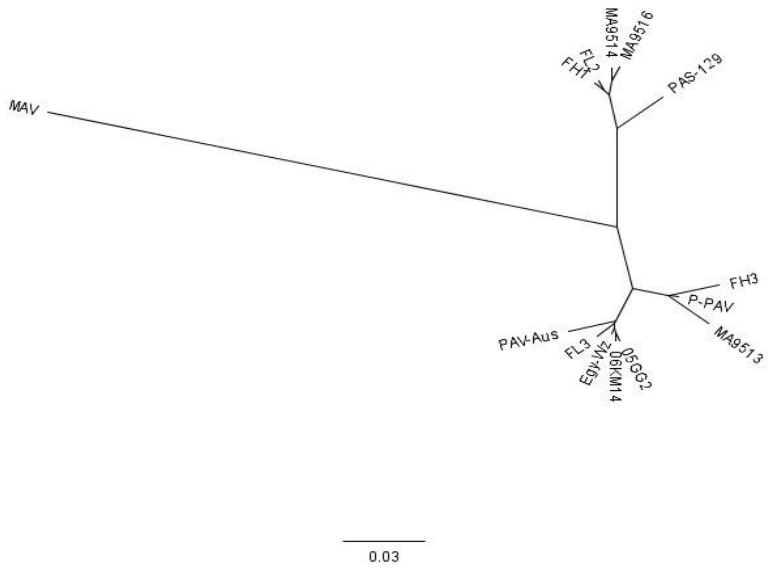


Figure5: Phylogenetic relationship of the of the BYDV-PAV of the Egyptian isolate Egy-Wz based on multiple nucleotide sequence alignments of the coat protein (CP) and other BYDV-PAV isolates available in GeneBank instructed by CLUSTAL W using Genious pro 8-0-5 Software.

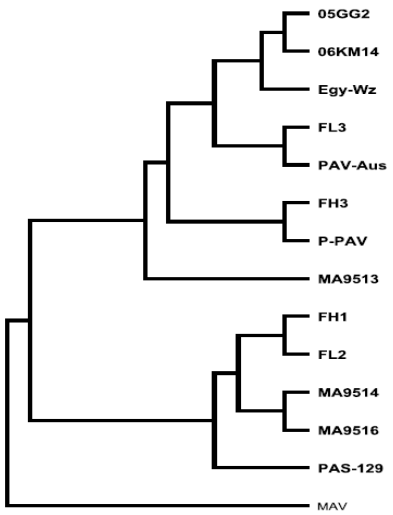


Figure 6: Phylogenetic relationship of the of the BYDV-PAV of the Egyptian isolate Egy-Wz based on deduced Amino acids sequence alignments of the coat protein (CP) and other BYDV-PAV isolates available in GeneBank instructed by CLUSTAL W using Genious pro 8-0-5 Software.

Discussion

Many genomic RNA nucleotide sequences of Luteoviruses have been published, Barley yellow dwarf virus and other closely related luteoviruses, Potato leaf roll virus (PLRV) and Beet western yellows virus (BWYV) [8,22 – 25]. The sequence data of genomic RNA available in GenBank[8, 26,27, 24], so, the developing of other primers became available and easier for detection of various strains of BYDV using specific primers.

In this study two viral genes were analyzed, and several primers were designed using two of the sequences of BYDV-PAV available in GenBank,BYDV-PAV acc.no NC002160 and BYDV-PAV acc.no. X0xeq7653, specific for two coding regions,polymerase gene located in (ORF1) and the coat protein located in (ORF3) respectively.BYDV-PAV isolate was reported and identified for the first time in New York in 1994 as a PAV variant, PAV-129as a PAS [28], but later it was reclassified as a different species from PAV in the genus luteovirus as a PAS serotype [3,29]. Serotypes PAV divideinto two species, PAV and PAS [29]. The two subgroups separated based on phylogenetic analysis of BYDV coat protein. Subgroup A which has isolates from 4 continents and include the Australian isolate the first isolate that was full sequenced. Subgroups B which include PAS isolate [30]. Thus, PAS- like isolates are found in Alaska,New York, France and Morocco [30].

The PAS species and PAV species of the Barley yellow dwarf virus (Luteoviridae) share hosts, vectors and distributions. With the sequence homology in the ORF1 and ORF2 of PAV-129 (BYDV-PAS) is 80% and 88%, respectively, comparing to ORF1 and ORF2 of all other PAV isolates and MAV, which are reached 97% identical between each other [31, 8]. In subgroup B isolates PAV-129 the coat protein sequence identity with subgroup A isolates is 86%.

The cDNA for ORF1 and ORF3 genes were cloned and sequenced. The clones that found to be presented the two-targeted genes were re-amplified using several new primers (BEgy-Wz1-2) and (BEgy-Wz3-4) whichwas designed based on the sequence of the Egyptian isolate BEgy-Wz of BYDV-PAV.

The PCR product for the coat protein was 603 bp in length and encodes for 200 amino acids, with molecularweight of 21.96 KDa, while for the partial polymerase gene it was 910 bp in length and encodes a predicted protein of 303 amino acids, with molecular weight 34.67 KDa. Vincent et al.,[27] demonstrated that BYDV isolates maybe different in some properties such as CP molecular weight.The BYDV-PAV (ORF3) encode for CP molecular weight of 22kDa [5]. The nucleotide sequence of the coat protein of the Chines BYDV-GAV isolate consists of 600 nucleotides [32].

In the phylogenetic tree for the polymerase gene P1 the sequence identity for the nucleotides and the amino acids sequence of the Egy-Wz isolate showed that the Egyptian isolate located in a cluster with PAV isolates.

The Coat protein nucleotide sequence alignment for BYDV-PAV isolate reported in 2002 [33, 34] with the present isolate showed identity of 99.8% (data not shown). The Cp sequence analysis for BYDV-PAV isolate reported in 2002 [33, 34] nucleotide alignment with the present isolate shows identity of 99.8% (data not shown). The phylogenetic relationships between the Egyptian isolate Egy-Wz isolated from wheat plant and other PAV and PAS isolates available in Genbank, for the nucleotides and the amino acidsof the coat protein for the Egy-Wz isolate indicated that the Egy-Wz located in the group A with the other PAV isolates(96 to 99.5% of amino acid identity). Despite the primers for CP gene designed from PAS isolate (group B) available in gene bank (X07653)the Egyptian isolate (Egy-Wz) showed only 84 to 86% amino acid similarity with group B.

In 1998 Mastariand his colleague [35] reported that PAV isolates belongs to subgroup A for the coat protein ranged from 93 to 100% at amino acid sequence identity, and the diversity of BYDV-PAV isolates was low despite the isolates were from different hosts, years, and locations, when it`s analyzed..

In addition, the Phylogenetic analysis showed that the Egy-WZ isolate was close to isolates 05GG2 and 06KM14 isolated from China which sharing also the same host plant species (Wheat plant) and had a strong similarity with FL3, PAV-AUS isolates from France and Australia isolated from different host species (Barley and ryegrass).These results showed that the Egyptian isolate belongs to PAV (group A) serotype and gave evidence that there is a strong correlation between the genetic diversity of the virus and host plant species and consequently it means that the host plants play a role in isolate selection.

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