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Research Article

Relationship of *Luteovirus* and PAV-Barley Yellow Dwarf Virus

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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) was targeted. Two sets of specific primers were 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 knot in length encodes a predicted polypeptide chain of 303 amino acids with an 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% sequence similarity 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 is 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%). BYDV is considered as a complex biological system, in spite of that in recent years furthers studies have been done and revealed significant information which facilitate the understanding of the molecular biology of the Barley Yellow Dwarf Virus (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. 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 plants infection.

Lines that have natural resistance for BYDV or tolerant at a significant level by the breeders are limited. Miller et al have completed the entire genome nucleotide sequence of barley yellow dwarf PAV serotype (BYDV-PAV) in 1988. The genome consists of 5677 nucleotides, which encodes six open reading frames (6 ORFs). 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. Genetic engineering has provided a powerful tool for virus resistance. Genetic resistance is safe for the environment and with reasonable cost; however BYDV natural resistance genes are few. 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. Besides the problems of conventional plant, breeding can be avoided by genetic engineering by introducing single resistance genes into

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commercial cultivars without carrying any linked deleterious genes. Transgenic plants have been obtained by introducing different artificial resistance genes. The mission of the artificial resistance gene is disturbing and interfering the viral replication cycle, and host-viral interaction. The viral coat protein is the first approach, which used very widely to be introduced to the host plant genome. 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.

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*.

MATERIALS AND METHODS

Virus source

Barley Yellow Dwarf Virus (BYDV) PAV was isolated previously from naturally infected wheat plants (*Triticum* sp) 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.

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.

For (ORF1) polymerase gene (*P1*), the primers were designed according to unpublished data BEgy3 (F) , BEgy4 (R) . For (ORF3) coat protein gene (*CP*) two primers were designed according to Miller, BEgy1 (F) , and BEgy2 (R), with restriction site for Bam H1 at the terminal 5'and aa tail (Table 1).

Table 1: 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	aaGAGCTCACATCGATGCGACT TGCTTT SacI		

Polymerase gene *P1* (ORF1)

RT reaction was carried out in 25 µl 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 tubes were sprint 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, 40 u/µl (Promega Cat # N211A), 1 µl of M-MLV RT 200 u/µl (Promega Cat# M1701),Nuclease–Free water to volume 25 µl. The tubes were mixed gently by flicking, and incubated for 60 min at 42°C.

PCR reaction

The PCR reaction mixture using specific BYDV-*P1* primers was prepared by using 2 of the resulting cDNA. The BYDV cDNA was transferred to a tube containing 10 of 1X polymerase chain reaction buffer (10 mm Tris-HCl, pH 8.3, 50 mm KCl, 0.01% gelatin (TaKaRa code# DRR100A), 2.5 mm $MgCl_2$, 200 M dNTPs, 10 pmol of BEgy3 (F) and BEgy4 (R) of forward and reverse primer respectively, 1 µ of Taq DNA polymerase (Ex Taq Takara). The amplification proceeded in the thermocycler (Uno II Biometra) at 94°C for 2 min, and through 40 cycles of 94°C for 30 s and 5°C for 30 s 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 (40 mm Tris–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. 200 M of dNTPs Mix was used. 2 µl of 25 mm $MgSo_4$ 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 vortexes. 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, 1 cycle 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-PAV PCR amplified product was directly cloned into linearized and thymidylated pGEM-T-easy vector according Promega kit cat#A1360. The constructs were transformed into E. coli competent cells according to Sambrook. 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 Genius Pro 8.0.5 software.

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

The clone that found to be presented cDNA for the ORF1 and ORF3 genes, 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 (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).

Table 2: The barley yellow dwarf virus isolates available in NCBI Gene Bank with their accession numbers, their country of origin and their host plant.

Isolate name	Country of origin	AC. 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	

PCR reaction

The PCR reaction has been done using the Premix Taq TaKaRa (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 45 cycle 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 35 cycle 94˚C for 30 s, 55˚C for 30 s and 72˚C for 1 min, 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 ORF1 and ORF3 were purified using Shanghai shenyou Biotechnology Co., LTd Kit # K0901 (100) as was mentioned previously. The PCR product of ORF1 has KpnI 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-Wz for 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. AcDNA 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).

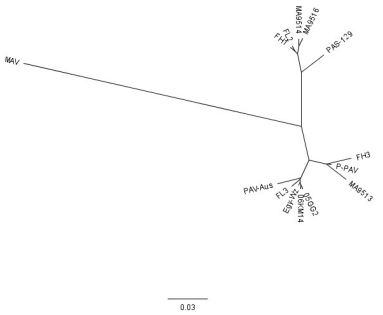

Figure 1: Phylogenetic relationship of the of the BYDV-PAV of the egyptian isolate Egy-Wz based on mµltiple nucleotide sequence alignments of the Coat Protein (*CP*) and other BYDV-PAV isolates available in GeneBank instructed by CLUSTAL W using Genius pro 8-0-5 software.

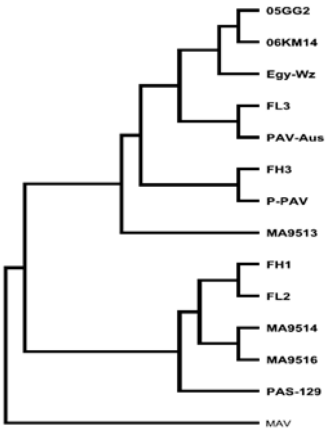

Figure 2: Phylogenetic relationship of the of the BYDV-PAV of the egyptian isolate Egy-Wz based on deduced Amino acids sequence alignments of the Coat Protein (*CP*) and other BYDV-PAV isolates available in GeneBank instructed by CLUSTAL W using Genius pro 8-0-5 software.

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 respectively. Multiple sequence alignments of nucleotide and deduced amino acids sequences were obtained with CLUSTAL W and Genius 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 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. 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 [6].

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). 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.

The Egyptian isolate coat protein sequences were 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. 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. 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 [7].

Phylogenetic relationships between isolates

The phylogenetic tree was constructed using Genius 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 [8]. 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. 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.

The PCR with specific primers

The resulting PCR fragments for ORF3 (*CP* coding region) was at the expected size about 603 bp, and for ORF1 (*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 [9-11].

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). The sequence data of genomic RNA available in GenBank, 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 Ac.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-129 as a PAS, but later it was reclassified as a different species from PAV in the genus *luteovirus* as a PAS serotype. Serotype PAV divides into two species, PAV and PAS. 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. Subgroup B which include PAS isolate. Thus, PAS- like isolates are found in Alaska, New York, France and Morocco.

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. 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) which was 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 molecular weight 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 demonstrated that BYDV isolates maybe different in some properties such as *CP* molecular weight. The BYDV-PAV (ORF3) encodes for *CP* molecular weight of 22 kDa [5]. The nucleotide sequence of the coat protein of the Chines BYDV-GAV isolate consists of 600 nucleotides.

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.

CONCLUSION

The Coat protein nucleotide sequence alignment for BYDV-PAV isolate reported in 2002 with the present isolate showed identity of 99.8%. The *CP* sequence analysis for BYDV-PAV isolate reported in 2002 nucleotide alignment with the present isolate shows identity of 99.8%. 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 acids of 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 Mastari and his colleague 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 its 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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