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MOLECULAR CHARACTERIZATION OF BARLEY YELLOW DWARF VIRUS (BYDV) ISOLATE T-UZB2 IN PROSO MILLET (*PANICUM MILIACEUM* L.) IN UZBEKISTAN

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SUMMARY

The Barley Yellow Dwarf Virus (BYDV) is one of the most economically significant viruses from the Luteoviridae family that infects most crop plants of the family Poaceae. The presented study sought to molecularly identify BYDV in proso millet (*Panicum miliaceum* L.) and evaluate the genetic diversity of its CP (coat protein) gene concerning other strains worldwide. Plant samples collected from proso millet fields in the Navoiy Region, Uzbekistan, incurred BYDV-PAV RT-PCR for the CP gene. The results authenticated the presence of a BYDV-PAV strain in the collected samples. The identified nucleotide sequence of BYDV-PAV isolate T-UZB2 proceeded to deposit in the GenBank database. BLASTN analysis of this sequence showed the highest genetic similarity (98.75%) with the Pakistan PAV-type isolate PK3 (JX473288.1). Phylogenic analysis and multiple sequence alignment revealed that four mutation events (268G>A, 440A>C, 450A>C, and 464C>T) involving the BYDV-PAV isolate Pk3 Pakistan have considerably contributed to the evolutionary history of PAV T-UZB2 Uzbekistan haplotype. SMV identification can help control pathogens and reduce their impact on the soybean crop in Uzbekistan.

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Keywords: Proso millet (*Panicum miliaceum* L.) survey, barley yellow dwarf virus (BYDV), RT-PCR, CP gene, phylogenic analysis, Navoiy Region, Uzbekistan

Key findings: The BYDV-PAV isolate detection in Uzbekistan occurred by RT-PCR in proso millet (*Panicum miliaceum* L.). The nucleotide sequence of the coat protein gene of this isolate reached recognition. Phylogenic analysis revealed its close relationship with the Pakistan PAV-type isolate PK3 (JX473288.1).

INTRODUCTION

Proso millet (*Panicum miliaceum* L.) is an annual herbaceous plant, a species of the Proso millet genus of the family Poaceae. Proso millet is one of Eurasia's most ancient cultivated plants, spreading from Southeast Asia. Its first cultivation dates back about 10000 years ago in China (Lu *et al*., 2009)**.** As an influential crop, its planting also covered a large hectarage in India, China, North Africa, the Middle East, Russia, and Ukraine. In Russia, proso millet growing is mainly in the arid regions of the Volga Region (Ilyin and Zolotukhin, 1986). In Uzbekistan, it is a widely grown repeated crop. Cultivating proso millet as a repeated crop allows for its harvest twice a year.

The Barley Yellow Dwarf Virus (BYDV) is one of four viruses that cause the most yield loss in cereal grain crops, severely affecting grain yield worldwide annually (Rybicki, 2015). BYDV's widespread worldwide results in significant yield losses (up to 80%), particularly where infection occurs early (McNamara *et al.*, 2020; Van-den-Eynde *et al.*, 2020). Reports also stated that the BYDV had caused yield losses of up to 25% in barley, 46% in wheat, and 15% in oats, and on average, 30% losses worldwide (Ordon *et al.,* 2009; Adhikari *et al.*, 2020).

Since its first report in 1953 (Oswald and Houston, 1951), it has remained a persistent and significant problem in cereal crop production. Barley yellow dwarf (BYD)-associated viruses constitute the most economically critical group of viruses infecting crop plants of the family *Poaceae*, including oats, wheat, barley, and rice, as well as pasture and wild grasses globally (Irwin and Thresh, 1990). BYDV (genus *Luteovirus*) and cereal (CYDV, genus *Polerovirus*) are single-stranded RNA viruses belonging to the family *Luteoviridae*, which can destructively affect more than 150 species of the *Poaceae* family (Bekele *et al.*, 2001; Balaji *et al.*, 2003).

The primary transmission of these viruses is mainly via the phloem through various species of aphid vectors and the different serotypes of BYDV classified based on the transmitting aphids (Thackray *et al.*, 2009). BYDV does not spread by mechanical means, flower pollen, or seeds, but only through aphids as virus vectors, with about 25 species of aphids listed in the literature, comprising chiefly of *Rhopalosiphum padi*, *Macrosiphum* (*Sitobion) avenae, Schizaphis graminum*, *Rhopalosiphum maidis*, and Acrosternum hilare (Deligöz *et al.*, 2011). The widespread studies of BYDV have covered several countries worldwide, i.e., BYDV in Iraq (El-Muadhidi *et al.*, 2001), Pakistan (Siddiqui *et al.*, 2011), Brazil (Parizoto *et al.*, 2013), Australia (Nancarrow *et al.*, 2014), Morocco (Bencharki *et al.*, 1999), and also recorded in Uzbekistan (Makhmudov *et al.*, 2023).

The gold-standard technique for identification of microorganisms is the amplifying and sequencing of DNA regions with sufficient variability to discriminate one species from another (Gohil *et al.*, 2023; Sherimbetov *et al.*, 2020a; Sherimbetov *et al.*, 2020b; Matniyazova *et al.*, 2022), including viruses (Trzmiel, 2017; Kim, 2023).

In previous research in Uzbekistan, the characteristics of the BYDV-PAV T-UZB1 isolate from wheat incurred assessment, carrying out molecular identification using the PCR method (Makhmudov *et al.*, 2023). In addition, *Zea mays* virus (Sobirova *et al.*, 2020, 2023), potato virus X (PVX) infecting potato plants (Fayziev *et al.*, 2020), and PPV infecting plum plants (Sattorov *et al.*, 2020) also bore identification. Moreover, studies have emerged

on preparing a specific serum for PVX from viruses and its diagnosis in potatoes (Jovlieva *et al*., 2024). The presented study sought to detect and molecularly identify BYDV in proso millet (*Panicum miliaceum* L.) in the Navoiy Region, Uzbekistan, and evaluate the genetic diversity of its CP gene concerning other strains worldwide.

MATERIALS AND METHODS

Sample collection

In September 2022, visually studying diseased symptoms of BYDV in proso millet fields began, collecting samples from infected plants in the Navoiy Region, Uzbekistan. The samples' storage comprised an Alpicool (Russia) refrigerator at -20 °C before proceeding to the laboratory. The excised plant leaves reached storage at -80 °C after placing them in labeled plastic bags to prevent viral RNA degradation.

Extraction and purification of total RNA

Leaf samples at 0.1 g sustained homogenization in liquid nitrogen before extracting the total RNA according to the manufacturer's protocol using Invitrogen™ PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA, https://www.thermofisher.com). Measuring the quantity and quality of total RNA continued, using a spectrophotometer NanoDrop Eight (Thermo Fisher Scientific, USA, https://www.thermofisher.com) before storing the RNA samples at -80 °C until used for RT-PCR.

Synthesis of the cDNA

The cDNA synthesis used SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, USA, https://www.thermofisher.com) and buffers in a final volume of 20 µl. From the total extracted RNA solution came the 5 ml mixed with 1 µl of 10 µM reverse primer Lu4 (Table 1), 4 µl 2, 5mM dNTP, and 3 µl nuclease-free water. Afterward, the samples were incubated at 65 °C for 5 min and quenched on ice for 3 min. The other reaction components' addition was in the following order: 4 µl of 5X PCR buffer, 1 µl 0.1 M dithiothreitol, 0.5 µl (20 U) of SuperScript IV reverse transcriptase, and nuclease-free water to give a reaction volume of 20 µl. The tubes received incubation for one hour and 55 minutes at 41 °C on a T960 PCR Thermal Cycler, followed by 10 min of heating at 700 °C to denature the enzyme.

Amplification by PCR

Reverse primer Lu4 and forward primer Lu1 could amplify a 530 bp PCR product within the viral coat protein gene of BYDV (Robertson *et al*., 1991). PCR reactions application used a Platinum hot start PCR 2x master mix (Thermo Fisher Scientific, USA, https://www.thermofisher.com) in a 25 µl volume, containing 4 µl 2X Master Mix, 0.5 µl of each primer (10 µM), 0.9 µl 25 mM MgCl, and 4 µl of the cDNA. The following thermocycling program continued for the PCR: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 1 min, annealing at 41 °C for 1 min, elongation at 72 °C for 20 min - 1 cycle, denaturation at 94 °C for 1 min, annealing at 41 °C for 1 min, elongation at 72 °C for 2 min - 45 cycles, and a final elongation of 72 °C for 5 min (Robertson *et al*., 1991).

Electrophoresis in agarose gel

Analysis of PCR products proceeded by electrophoresis on a 2% agarose gel prepared in 1x tris-borate-EDTA buffer (TBE, Thermo Scientific) stained with ethidium bromide. The addition of 2 µl of DNA Gel Loading Dye (6X) and 1.3 µL of 100 bp DNA Ladder continued to

Table 1. Primers used in this study.

Target region	Name of the primers	Sequence (5′-3′)	Product size (bp)	Source
CP	Lu1-R	CCAGTGGTTRTGGTC	530	Robertson *et al*. (1991)
CP	Lu4-F	GTCTACCTATTTGG		

the first well of agarose gel, then 10 µl PCR products with 3 µl of DNA Gel Loading Dye (6X) proceeded to subsequent agarose wells. Electrophoresis ensued using horizontal electrophoresis system SE-1 (Helicon, Russia, https://helicon.ru) at 100V for 100 min. PCR product visualization uses UV light and photography using a gel document imaging system BK-AG100 (Biobase, China, https://www.biobase.com/product-list/gel-document-imaging-system).

Sequencing of PCR products

The excising of one PCR band used a razor blade, then transferred to a 1.5 ml centrifuge tube and purified according to the manufacturer's instructions using the PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, USA, https://www.thermofisher.com). Cycle sequencing reaction application utilized the BigDye® Terminator v 3.1 kit (Thermo Fisher Scientific, USA, https://www.thermofisher.com). Cycle sequencing reaction consists of ddH2O-3.5 µl, BigDye- 1 µL, 5x Seg.buffer-2 µl, sequencing primer - 0.5 µl, and purified PCR product - 2 µl. Lu1 and Lu4 primers were materials for sequencing. The following thermocycling program commenced for the cycle sequencing reaction: initial denaturation stage at 96 °C for 1 min; denaturation at 96 °C for 10 s, annealing at 41 °C for 10 s, and elongation at 60 °C for 3 min, repeated for 45 consecutive cycles. Storing the product of the sequence reaction had a temperature of 4 °C. The sequencing reaction products purification from fluorescently labeled terminator nucleotides used the Dynabeads Sequencing Clean-Up Kit (Thermo Fisher Scientific, USA, https://www.thermofisher.com).

Separation and analysis of DNA sequence reaction products by capillary gel electrophoresis with laser-induced fluorescence detection occurred on Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific) Sequence alignments and phylogenetic analyses: Snap Gene 5.3.1 program to edit the raw data obtained from the DNA sequence analysis system. Nucleotide sequences examination employed the BLASTN program (basic local alignment search tool nucleotide-nucleotide, http://www.ncbi.nlm.nih.gov/BLAST/) (Camacho *et al.*, 2009). Multiple sequence alignments ensued by ClustalW. The Phylogenetic tree construction by the Neighbor-Joining (NJ) method used the Kimura 2-parameter model, with statistical support performed by the bootstrap method of interior-branch test for phylogenetic trees (Kimura, 1980) (Figure 1). Bootstrap test of phylogeny and visualization of the final phylogenetic tree engaged the MEGA11 software package using the Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al*., 2021).

RESULTS

The monitoring and identification of BYDV in proso millet (*Panicum miliaceum* L.) fields commenced in September 2022 at the Navoiy Region, Uzbekistan. During the study, proso millet plants showed typical symptoms of virus infection, observing yellowing of leaves, dwarfing, and chlorotic strip patterns on the leaves (Figure 2). In the presented research, RT-PCR application amplified the partial sequence of the CP gene on the proso millet samples. PCR products with universal Lu one/Lu four primers resulted, as predicted, 530 bp long (Figure 3). BLAST analysis in the NCBI database showed that the investigated sequence belonged to the BYDV-PAV serotype.

The BLAST analysis of Uzbekistan revealed that isolate T-UZB2 was evidently against a nucleotide sequence database, indicating that the isolate was similar to other isolates at rates ranging from 89.87% to 98.75%. This isolate showed the highest genetic similarity (98.75%) with the seven Pakistani PAV-type isolates, i.e., PK3(JX473288.1), Chk04 (KT222667.1), M21 (KT198982.1), PK2 (JQ811489.1), PK4 (JX473287.1), FSD(GU247981.1), and PSW2 (GU247976.1), and one Iranian PAV-type isolate IR-PAV (AY450454.1). However, the lowest similarity rate (89.87%) appeared with the Tunisian isolate PAV-TN5 (JX402457.1).

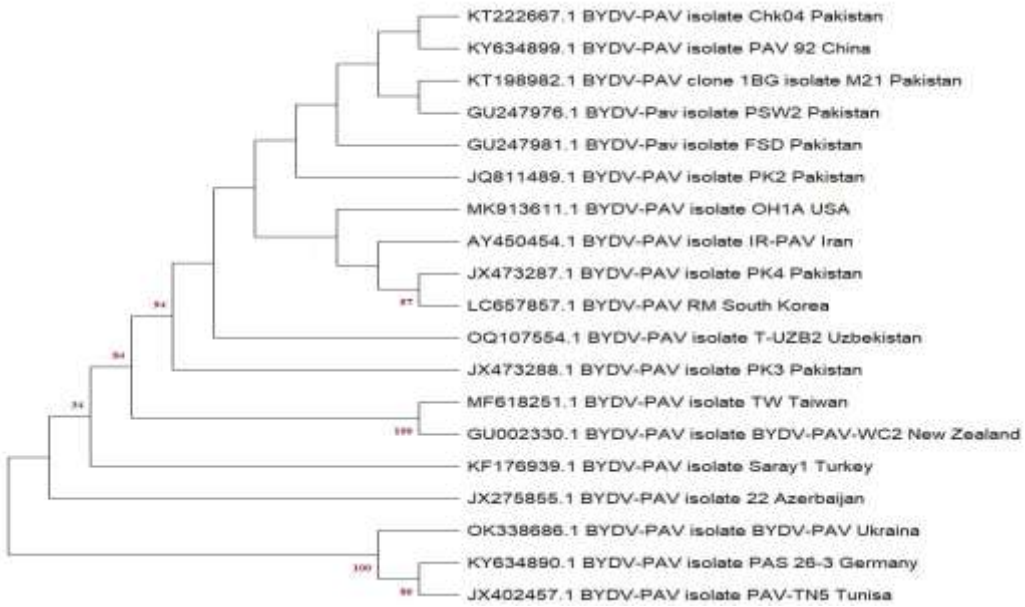

Figure 1. Neighbor-joining (NJ) tree for the CP gene of BYDV-PAV isolates. Numbers above branches are bootstrap support values (when higher than 50%) based on 1000 replicates.

Figure 2. BYDV disease symptoms in proso millet (*Panicum miliaceum)* field of Navoiy region.

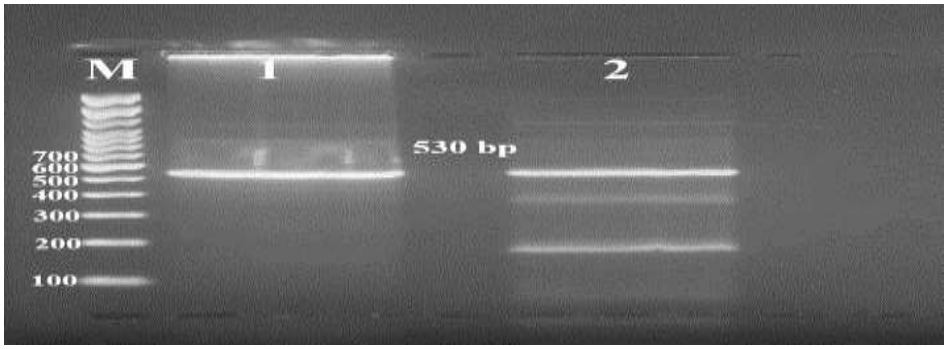

Figure 3. Electropherogram of RT-PCR performed with universal Lu 1/Lu 4 primers in proso millet samples from Navoiy region; M - DNA 100 bp DNA Ladder; 1–2 *Panicum miliaceum* samples.

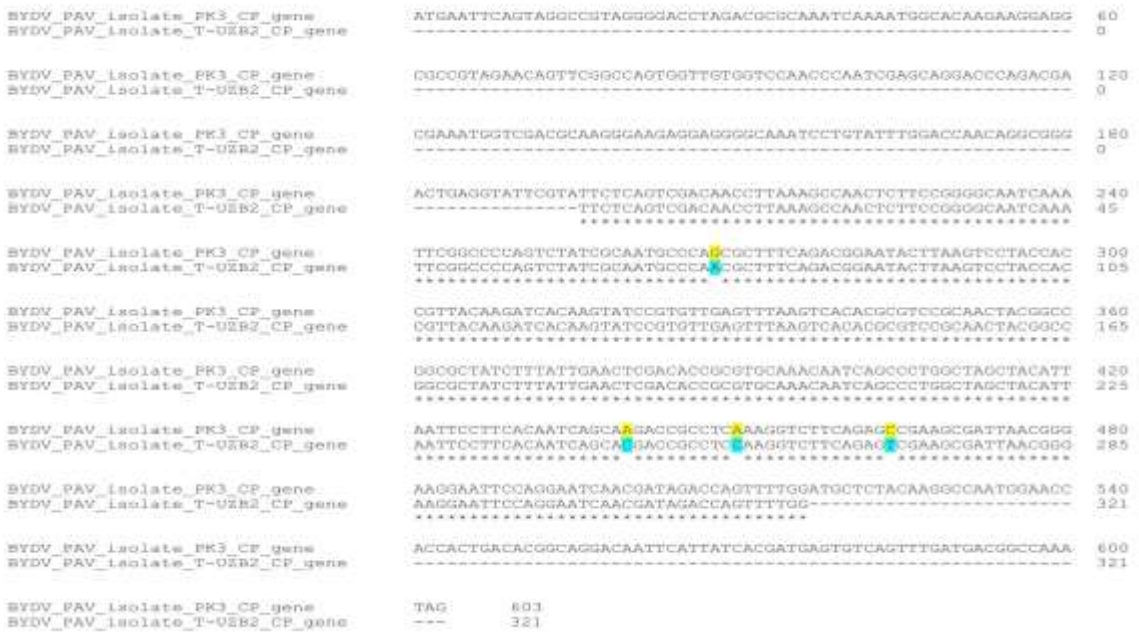

Figure 4. Alignment of CP gene sequences of BYDV PAV T-UZB2 isolate (Uzbekistan) and BYDV PAV PK3 isolate (Pakistan).

For phylogenetic analysis, the partial nucleotide sequence of the CP gene of Uzbekistan T-UZB2 OQ107554.1 isolate reached a comparison with 19 strains of the BYDV-PAV available in the GenBank. According to the phylogenetic tree generated with 19 different homologous sequences of BYDV-PAV, the BYDV PAV T-UZB2 isolate sequence showed clustering with seven Pakistan isolates, PK3 (JX473288.1), Chk04 (KT222667.1), M21 (KT198982.1), PK2 (JQ811489.1), PK4 (JX473287.1), FSD(GU247981.1), and PSW2 (GU247976.1), and one each for the Iranian PAV-type isolate IR-PAV (AY450454.1), and the Chinese isolate PAV 92 (KY634899.1).

Phylogenic analysis and multiple sequence alignment (using ClustalW) strongly suggest that four mutation events, i.e., 268G>A, 440A>C, 450A>C, and 464C>T, involving the BYDV-PAV isolate Pk3 Pakistan, have considerably contributed to the evolutionary history of PAV T-UZB2 Uzbekistan haplotype (Figure 4).

Comparative analysis of T-UZB2 isolate, with a deduced coat protein of Pk3 isolate, showed that three mutations, i.e., 268G>A, 440A>C, and 464C>T of T-UZB2 isolate were the missense mutations as a result in amino acid substitutions in the protein-coding region (A90T, K147T, and A155V) (Figures 5 and 6). In that, the one mutation (450A>C) emerged as the silent mutation that does not affect the encoded amino acid sequence of the coat protein.

Alignment of the coat protein of the BYDV-PAV Pk3 isolate with the T-UZB2 isolate and the results of pair-wise superposition of A90T, K147T, and A155V amino acid substitutions concerning luteovirid coat protein secondary structure elements appear in Figure 7 (Byrne *et al.*, 2019). Forming of the luteovirid capsids from CP subunits with a canonical jellyroll fold comprised two opposing β sheets, each containing four antiparallel β strands (βB-βI). This core structure contained two helices (α1 and α2) decorating it. An analysis of the pair-wise superposition of amino acid substitutions revealed that A90T substitution localized in the α1 helix. Meanwhile, K147T localization surfaced in the loop between the βF and βG, and A155V localized α2 helix of the coat protein.

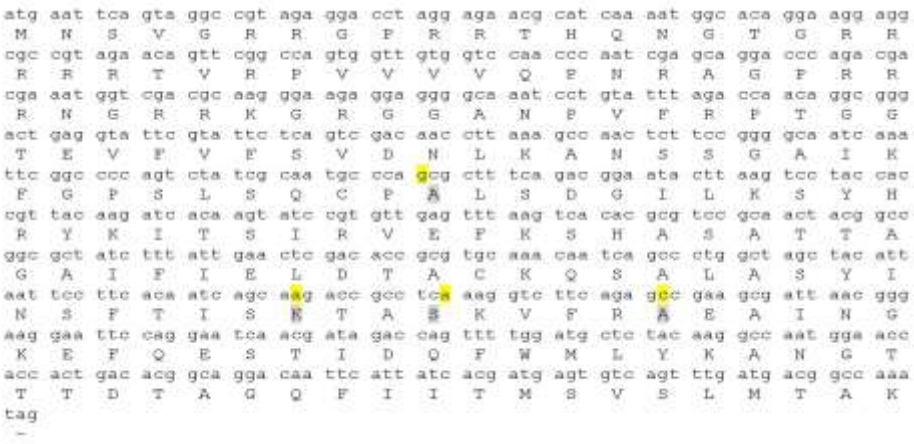

Figure 5. Deduced coat protein of Pk3 isolate (Pakistan).

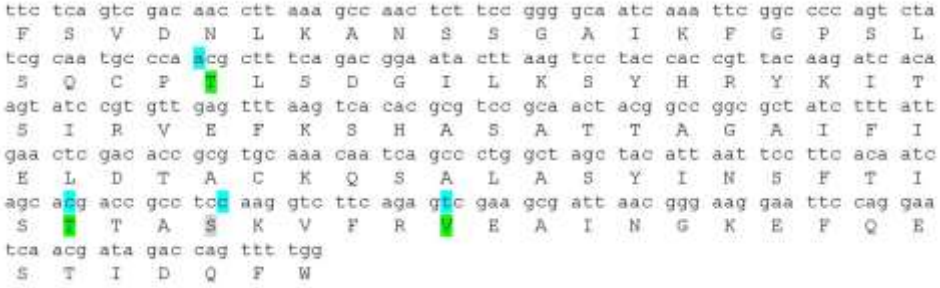

Figure 6. Deduced coat protein of T-UZB2 isolate (Uzbekistan).

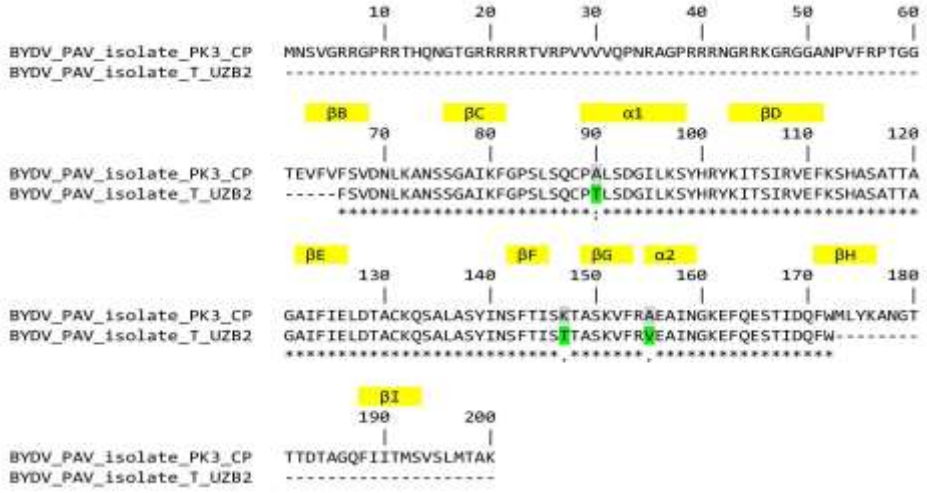

Figure 7. Alignment of coat protein sequences of BYDV PAV T-UZB2 isolate (Uzbekistan) with BYDV PAV PK3 isolate (Pakistan) and pairwise superposition of amino acid substitutions in relation to coat protein secondary structure elements. Secondary structure elements are those highlighted by yellow boxes.

DISCUSSION

BYDV is widespread globally and infects cultivated and wild plant species from the Poaceae family. In particular, it causes severe yield losses in barley, wheat, and sorghum (Choudhury *et al.*, 2018; Zhapayev *et al*., 2023). Phylogenetic analysis of T-UZB2 isolate revealed similarities and differences with other strains worldwide. In the phylogenetic tree analysis, it is visible that the TUZB2 isolate OQ107554.1 separated from the proso millet of the Navoiy Region and showed 98.75% similarity with the Pakistan BYDV-PAV Chk04 (KT222667.1) isolate.

The promising results were also consistent with the speculation that BYDV originated in the United States before spreading to Asia through China (Wei *et al.*, 2023). Multiple migration pathways of BYDV from the USA and China to other regions were also distinct, indicating that the USA and China became the critical hubs for the global spread of this pathogen. Results of this research were also in line with previous studies conducted revealing that after spreading from the United States to China, it further spread from China to Pakistan and Iran, then other countries of South Asia to Uzbekistan, and other countries of Central Asia (Wei *et al*., 2023).

Therefore, the barley yellow dwarf virus spread to the countries of South Asia and Central Asia, and the population size increased dramatically. Under different selective pressures from various agricultural management practices, habitats, resistant cultivars, and agrochemicals, the evolutionary pattern of the barley yellow dwarf virus was unique to the region and host.

Proso millet (*Panicum miliaceum* L.) is a highly nutritious cereal grain used for human consumption, and feed for animals, including ruminants, poultry, pet birds, and ethanol production. Unique characteristics, such as drought and heat tolerance, make the proso millet a promising alternative cereal. As a result of the recent strengthening of proso millet cultivation in Uzbekistan, it has also become a hazardous weed in other crop fields. The lack of seed dormancy helps it to spread easily and invade maize, wheat, and other crop fields. Pasztor *et al.* (2020) reported that proso millet could be a virus reservoir in different crop fields. These viruses can also cause severe diseases in wheat and other cereals, and their presence in weed form implies a potential infection risk. The RT-PCR assay and molecular identification of BYDV used in this study proved useful in the molecular diagnosis of the BYDV infection in proso millet in Uzbekistan to uncover hidden virus reservoirs in crop fields. Likewise, the methods can advise the timely conduct of control strategies to prevent the emergence of these viral diseases in plantations.

Also, the determined nucleotide sequence of the CP gene of the T-UZB2 genotype and its deduced coat protein could have an essential implication related to the biological properties of this virus. BYDV capsid comprised two forms of CP, the major P3 protein (ORF3) and the minor P3/P5 protein (ORF3 + ORF5), which is the final product of a translational readthrough of P3 (Wang *et al.*, 1995). In BYDV, the P3/P5 is responsible for insect transmission, requiring a long-distance movement in phloem tissue in different crop plants (Taliansky *et al.*, 2003).

Mutations demonstrated that the coat protein of squash leaf curl - China virus (SLCCNV) rendered the virus more competitive in transmission by particular species of whiteflies (Pan *et al.*, 2020). The investigation of the key residues responsible for the change in whitefly transmission also revealed that a S147T substitution was sufficient to induce the whitefly transmission of a virus between two cryptic species of whitefly, i.e., Asia-I (predominant in Guangxi province, China) and Asia-II (predominant in Lahore, Punjab province, Pakistan) (Masood *et al.*, 2017).

It also supported the remarkable hypothesis of an association between insect vector species and virus genotypes. In addition, the viral CP has become the factor triggering resistant responses of host plants against viral infections (Beachy, 1999). Therefore, it is possible that in this study, amino acid substitutions in the CP protein-coding region could have an influence on some biological properties of the BYDV, viz., insect transmission, virus movement in the phloem,

or variations in triggering resistant responses in plants, facilitating virus specialization on local aphid vectors and host plants in the Uzbekistan region. This phenomenon also opens new perspectives requiring further research to determine a functional consequence of missense mutations identified in the presented study.

Determination of the CP gene sequence of the virus isolates is also vital to finding an appropriate control strategy. The most environmentally feasible control strategy to combat BYDV infection is developing tolerant wheat and proso millet cultivars. Some natural resistance genes against BYDV have also been evident, such as the *Yd2* gene in barley (Collins *et al.*, 1996), *Bdv1* in wheat (Singh *et al.*, 1993), and *Ryd4Hb* from *Hordeum bulbosum* (Pidon *et al.*, 2023). Notably, besides these plants, the BYDV virus also infects *Festuca* spp., *Elytrigia repens*, *Echinochloa crus-galli*, *Lolium multiflorum, Arrhenatherum elatius,* and Bromus spp., *Avena sativa, Avena fatua,* and *Zea mays* (Jarošová *et al.*, 2013).

However, rapidly evolving viruses may circumvent these natural resistance genes (García-Arenal and McDonald, 2003; McDonald and Linde, 2002; Jewehan *et al*., 2022). These factors also enhance the complexity of plant-virus interactions. They recommend further research to understand the molecular evolutionary dynamics better and elucidate the molecular-genetic aspects of the 'battle' for survival between viruses and their host plants.

CONCLUSIONS

Based on the results, the BYDV-PAV strain detection was evident in the proso millet (*Panicum miliaceum* L.) plant samples. The studied nucleotide sequence of BYDV-PAV received the name T-UZB2 isolate. The T-UZB2 isolate of BYDV-PAV strain isolated from proso millet plants analyzed phylogenetically, proved closely related to the seven Pakistani PAV-type isolates (PK3, Chk04, M21, PK2, PK4, FSD, and PSW2) and one Iranian (PAV-type isolate IR-PAV). The RT-PCR assay and molecular identification of BYDV were beneficial in detecting BYDV infection in proso millet in Uzbekistan to reveal hidden virus reservoirs in crop fields and timely conduct of control strategies to prevent the emergence of viral diseases in plantations.

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