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شناسایی مولکولی و آزمون بیماریزایی سویه جو از ویروس کوتولگی گندم در جو و گندم*

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چکیده

بیماری زردی و کوتولگی گندم و جو از عوامل محدودکننده تولید این محصولات در ایران و برخی نقاط دنیا است. سویه های گندم و جو ویروس کوتولگی گندم (*Wheat dwarf virus*, WDV) مرتبط با این بیماری هستند. اگرچه اختصاصی بودن میزبان این استرین ها در گندم و جو بحث برانگیز است. در این تحقیق، یک واریانت جدید از WDV از جو در جنوب غرب ایران جداسازی و توالی یابی شد. ژنوم کامل این جدایه ۲۷۳۲ نوکلئوتید طول دارد و متشکل از چهار چارچوب ژنی است. بررسی توالی کامل ژنوم بیانگر شباهت بالای این جدایه (۸۳.۷ تا ۹۷٪ شباهت) با جدایه WDV از جو بود و به عنوان یک واریانت از این ویروس تعیین شد. بررسی فیلوژنتیکی نشان داد که این جدایه در کنار سایر جدایه‌های اروپائی این ویروس از جو در یک زیرگروه به همراه جدایه دیگری از ایران قرار می گیرد. همسانه بیماریزای این ویروس از ساختار ۱.٤ برابر ژنوم در یک ناقل دوتایی تهیه شد. اگروانیوکولیشن دی‌اِن‌اِی همسانه سازی شده در هر دو گیاه گندم و جو ایجاد علایم زردی و کوتولگی کرد. با استفاده از این سیستم، جدایه جو ویروس کوتولگی گندم با راندمان مشابه، گندم و جو را آلوده می‌سازد. این همسانه بیمارگر، جهت غربالگری جو و گندم، شناسایی ژن/های مقاومت و تعامل ویروس_میزبان قابل استفاده می‌باشد.

کلیدواژه: اگروانیفکشن، جمینی‌ویروس، ویروس های غلات، تکثیر به روش دایره غلطان

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Molecular identification and infectivity assay for a barley strain of wheat dwarf virus in barley and wheat*

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Abstract

Yellowing and dwarfing disease in wheat (*Triticum aestivum*) and barley (*Hordeum vulgaris*) plants is a major constraint for yield production worldwide. Wheat and barley strains of wheat dwarf virus (WDV) were found to be associated with the disease in Iran and some parts of the world. However, host specificity of wheat and barley strains of WDV is controversial. In this study, we isolated and sequenced a new variant of WDV from barley plants in the southwest of Iran. The full-length genome of this isolate comprises 2,732 nucleotides with four open reading frames. Sequence analysis of the full-length genome revealed that this isolate is similar (87.3 to 97% identity) to the barley isolates of WDV and was considered as a new variant of the virus. Phylogenetic analysis indicated that this isolate is grouped with other barley isolates of WDV from Europe in a distinct sub-group with a barley isolate from Iran. An infectious clone of WDV was constructed in a binary vector using a partial dimer of the full-length genome. Agroinoculation with the cloned viral DNA resulted in yellowing and dwarfing symptom in both wheat and barley plants. Using this system, the barley isolate of WDV infects wheat and barley with similar efficiency. This infectious clone can be used for screening both wheat and barley plants for resistance/susceptibility, identification of host resistance gene/s and investigating the virus-host interaction.

Keywords: Agroinfection, Cereal viruses, Geminivirus, Rolling circle amplification method

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Introduction

Yellowing and dwarfing disease in wheat (*Triticum aestivum*) and barley (*Hordeum vulgaris*) is economically important and is caused by a number of viral agents. Among them barley yellow dwarf viruses (BYDV) from the family *Luteoviridae* is the most common virus associated with the disease (Rastgou *et al.*, 2005), whereas wheat dwarf virus (WDV) has also been reported from various regions of Iran and throughout the world (Behjatnia *et al.*, 2011; Köklü *et al.*, 2007; Kvarnheden *et al.*, 2002; Schubert *et al.*, 2007; Lotfipour *et al.* 2013; Ghodum Parizpour *et al.* 2016), which makes the yellowing and dwarfing disease more complex.

WDV is a member of the genus *Mastrevirus* (family *Geminiviridae*) which contains a monopartite single-stranded (ss) and circular DNA genome and infects monocotyledonous plants from the family *Poaceae*. The genome of WDV contains four open reading frames (ORFs) encoding for the movement protein (MP, V1) and the capsid protein (CP, V2) on the virion sense strand and two replication associated proteins, Rep and Rep A on the complementary strand (Brown *et al.*, 2012). WDV is transmitted by leafhoppers (*Psammotettix striatus, P. alienus* and *P. provincialis*) in a circulative, nonprogagative manner to barley, wheat, oats, rye and many kinds of wild grass (Lotfipour *et al.* 2013; Lemmetty and Huusela-Veistola 2005) Infected plants show yellowing symptoms, produce more tillers and are dwarfed. This has resulted in shriveled grains and reduced yield (Nygren *et al.*, 2015).

Two strains of WDV, wheat strain and barley strain, were reported according to their preferential occurrence in wheat or barley in the field. Their genomes show a high nucleotide similarity, 78 to 86% (Köklü *et al.*, 2007; Lotfipour *et al.*, 2013; Schubert *et al.*, 2007). Both strains have a wide and partially overlapping host range in the family *Poaceae* (Lindsten & Vacke, 1991). Six groups (A–F) for WDV isolates have been suggested based on the sequence similarity between isolates and their phylogenetic relationship (Muhire *et al.*, 2013) in which groups B and D have only been reported from Iran. Group F includes only WDV isolates from Iran and virus strains in this cluster diverged earlier than the other isolates of WDV-Wheat (Ghodum Parizpour *et al.* 2016).

For monocot-infecting geminiviruses, naked viral DNA was not infectious when rubbed onto leaves or injected into various plant tissues (Fang and Dale, 1992). This indicates that delivery of the viral DNA requires a suitable method. The technique of "agroinfection" has been used for the introduction of cloned viral DNA into plants (Grimsley *et al.*, 1986) and has provided an effective infection which has been parallel to that of insect vector inoculation. In this system the infectious clone need to be made as a dimer or partial dimer of the virus genome in a binary vector. The infectious clone can also be delivered into host plants/cells using particle bombardment (Briddon *et al.*, 1998; Suarez-Lopez and Gutierrez, 1997). Agroinfection method has been successfully used for infecting wheat and other host plants with an infectious clone of WDV (Ramsell *et al.* 2009).

The widespread occurrence and genetically unique isolates of WDV in Iran made a challenge for breeders to produce plants with tolerant or resistant traits. Providing breeders a simple and efficient method of virus inoculation can accelerate their studies. In addition, infectivity of wheat and barley strains of WDV in wheat and barley plants is controversial. The aim of this study was to isolate WDV from barley plants and to produce an infectious clone for WDV to fulfill Koch's postulates in host plants. This infectious clone can be used for further investigation of the screening and identification of host resistance gene/s by breeders and virus-host interaction.

Materials and methods

Plant material

Infected barley plants with yellowing and dwarfing symptoms were collected in 2015 from a barley field in Chahar Mahal province (Mamooreh's fields), in the southwest of Iran. For glasshouse experiments, wheat (cv. Sardari) and barley (cv. Makoye) were grown in pots containing loamy sand, vermiculite, and coco peat (1:1:1) and maintained under 14/10-h light/dark periods at 22±4 °C.

Virus detection, cloning and sequencing

Total DNA was extracted from leaf tissues of barley plants by a method described before (Rouhibakhsh *et al.*, 2008). For rolling circle

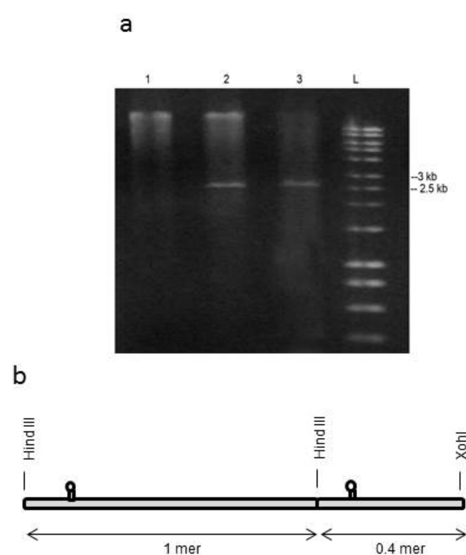

Fig. 1. Electrophoretic pattern of the amplified DNAs by RCA and digested by *Hind* III enzyme (A). Lane 1 shows the amplified DNA without digestion, lanes 2 and 3 show a *Hind* III DNA fragment released from the RCA amplified DNA. L= DNA ladder. A schematic shows the 1.4 mer DNA construct of WDV (B) that was cloned into pBin20 and designated pBin20-1.4WDV. The position of restriction enzymes and step loop are shown on the construct.

amplification (RCA) of circular DNA molecules by Phi29 DNA polymerase (TempliPhi kit, General Electric Healthcare), two hundred nanograms of the DNA samples were mixed with 5 µl of the provided sample buffer and denatured at 95 ºC for 3 min. Then, 0.2 µl of the enzyme and 5 µl of the reaction buffer were added and samples were incubated at 30 ºC for 18 h. The amplified DNAs were digested with *Hind* III to release the full-length DNA of the virus. DNA fragments were separated in 1.2 % agarose gels and visualized by staining with ethidium bromide. The DNA fragments of ~2.7 kbp were purified from the agarose gel using a PCR clean-up Gel extraction kit (GF-1, Vivantis). The purified fragments were ligated into *Hind*III site of the pBluescript II SK (+) (Stratagene) and then transformed into *Escherichia coli* (strain DH5α) cells. Bacterial colonies were screened by PCR using universal primers and inserted fragments in three individual clones were sequenced (Macrogen, Korea) by primer walking technique using M13F/M13R universal primers and specific internal WDV primers. One of these clones was designated pBSK-0.1WDV. This clone was used as the DNA template to construct the infectious clone of WDV.

Sequences analysis

The consensus sequence out of the paired sequences for pBSK-0.1WDV, isolated from an infected barley plant with yellowing and dwarfing symptoms, was compared to available sequences in the GenBank database using BLAST software (www.ncbi.nlm.nih.gov/gen bank). To obtain the full-length sequence, a contig of the sequences was performed using BioEdit software (Ver. 7.2.5, Carlsbad CA). This sequence was used to compare with other available sequences in the GenBank database for WDV isolates from various regions of the world.

For phylogenetic analysis, a multiple sequence alignment was made using the program AlignX (BioEdit) with the ClustalW algorithm (Higgins *et al.*, 1994) and then a neighbor-joining method was used to construct the phylogenetic tree with 1000 bootstrap replicates using MEGA6 (Tamura *et al.*, 2013). To estimate the evolutionary divergence between the obtained WDV sequence from this study and other WDV sequences available in GenBank, the maximum composite likelihood model (Tamura *et al.*, 2004) was used and analyzed by MEGA6 (Tamura *et al.*, 2013). To include a wide range of WDV isolates, we selected WDV isolates from wheat and barley from all countries that WDV has been reported (Table S1).

Construction of infectious clone

To construct a head-to-tail partial dimer of WDV, a 1,058 bp DNA fragment was released from pBSK-1.0WDV through digestion of this clone with *Hind*III/*Xho*I enzymes. This fragment was sub-cloned into the corresponding sites of a binary vector, pBin20 (Hennegan and Danna 1998), to obtain the pBin20-0.4WDV construct. The full-length *Hind*III monomeric DNA fragment was released from the pBluescript II SK-1.0WDV construct through digestion of this clone with *Hind*III and then sub-cloned into the corresponding site of pBin20-0.4 WDV construct to create a 1.4 mer DNA construct of WDV that designated pBin20-1.4WDV (Fig. 1B). The correct orientation of this construct was confirmed by *Xho*I digestion.

The resulting construct was introduced into *Agrobacterium tumefaciens* strain C58 by electroporation (50 µF, 1.8 kV, 150 Ω) with a Gene Pulser apparatus (Bio-Rad, Germany) according to the manufacturer's specifications.

Virus infectivity assay

A. tumefaciens cells harboring pBin20-1.4WDV were grown at 28°C for 48 h. Bacterial cells were collected and resuspended in the infiltration buffer (10 mM morpholineethanesulfonic acid [MES], 10 mM $MgCl_2$, and 100 µM acetosyringone) and then approximately 30 µl of the cell suspension (OD_{600} = 0.2) was injected into two to three spots into the stem of each wheat and barley seedlings at the four-leaf stage, as described previously (Dale *et al.*, 1989). For control, plants were mock inoculated with bacterial cells containing pBin20 vector.

The agroinoculated plants were monitored for symptom appearance from 14 days after inoculation. Developing leaves were sampled from these plants at 21 dpi and total DNA was extracted and analyzed for the presence of the viral genome by PCR. Infectivity rate was calculated by the ratio of the number of PCR positive plants over the number of inoculated plants. This experiment was repeated to obtain an average for the infectivity rate in both wheat and barley plants.

Results

Detection and sequence analysis of WDV

DNA extracts obtained from barley plants showing yellowing and dwarfing symptoms were analyzed by RCA for the presence of WDV. Digestion of RCA products by *Hind* III released a DNA fragment of the expected size (approximately 2,700 bp) from two symptomatic plants (Fig. 1A).

Three individual clones from an infected plant were sequenced and a contig of these sequences (2,732 nt) was obtained. Searching GenBank database, the sequence of this isolate was similar to the other isolates of WDVs from barley in the genus *Mastrevirus.* The genome of this isolate contains four OFRFs including replication associated proteins (Rep A and Rep) on the complementary-sense strand, a movement protein (MP) and a coat protein (CP) on the virion-sense strand. Similar to the other members of mastreviruses, a small (171 nt) and a large (402 nt) intergenic region (IR) are present in the genome. The large IR contains a stem-loop structure with the conserved nonanucleotide sequence, TAATATT/AC, which is part of the intergenic region in geminiviruses (Heyraud-Nitschke *et al.*, 1995). The nucleotide sequence data has been deposited in GenBank under accession number KX889118.

Sequence analysis and phylogenetic analysis of WDV-Bar[IR]

Sequence alignment of the obtained full-length genome of WDV with the other WDV isolates from various regions of the world showed a high similarity to that of barley isolates from Iran (97.6%, FJ620684), Czech Republic (89%, FJ546178), Germany (88.9%, AM921993) and Poland (88.8%, KM079155), hereafter called WDV-Bar[IRI]. Pairwise distance for the selected WDV full-length sequences (Table S1) was calculated using maximum composite likelihood method (Tamura *et al.* 2004). The full-length sequence of WDV-Bar[IRI] is close to barley (87.3 to 97.6% identity) and wheat (85.2 to 90.7% identity) isolates of WDVs (Table 1).

The deduced amino acids for the ORFs of WDV-Bar[IRI] were compared to the available sequences in the GenBank database which showed a high similarity (79 to 98% identity) to their counterparts in WDV isolates from barley. A high level of conservation was observed for all four proteins including Rep A (81 to 97%), Rep (79 to 98% identity), MP (86 to 96% identity) and CP (86 to 98% identity). The highest nucleotide sequence variation (72.4 to 97.4% identity) was observed for the LIR region (Table 1).

A dendrogram made by phylogenetic analysis (Fig. 2) of the full-length genome sequence of WDV-Bar[IRI] and other WDV isolates from wheat and barley from various geographical regions (Table S1) revealed two separate groups for barley (Fig. 2, Group II) and wheat (Fig.2, Group I) strains. The barley isolate from this study, WDV-Bar[IRI], was placed in the barley group (Fig. 2, Group II), but in a distinct sub-group with another barley isolate from Iran (FJ620684). In the barley group, WDV from Germany (AM942044) and Ukraine (FN806787) have been isolated from wheat and in the wheat group, isolates from China

Table 1. Percent nucleotide (nt) and amino acid (aa) identities of WDV-Bar[IRI] with those of other WDV isolates. They have been sorted based on the level of complete sequence identity.

	Complete	MP		CP		Rep A		Rep		LIR
WDV isolate	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt
FJ620684-B-IRI	97.6	98.1	96.6	98.1	97.3	98.2	98.5	98.6	81.6	97.4
JN791096-W-IRI	90.7	94.3	90.7	92.2	92	94.8	95.7	92.9	98.1	80.5
FJ546178-B-CZE	89	92.7	93.1	86.5	88.2	91.2	95	91.1	96.5	92.7
FN806787-B-UKR	89	91.4	91.9	85.7	87.3	92.7	95.7	91.8	96.9	92.3
AM921993-B-DEU	88.9	91.9	93.1	86.4	87.7	91.5	95	91.2	96.5	92.2
AM989927-B-BGR	88.8	91.5	93.1	85.7	86.9	92.2	94.2	91.6	95.7	90.5
AM296018-B-DEU	88.8	91.4	91.9	86.4	87.7	91.5	95	91	96.5	92.6
KM079155-B-POL	88.8	91.9	93.1	86.1	87.7	91.3	95	91	96.5	93.1
AM942044-B-DEU	88.7	91.9	93.1	86.1	87.7	91	94.2	90.8	95.7	92.3
FM210034-B-HUN	88.5	91	91.9	86.5	87.7	90.8	95	90.5	96.1	92.3
FJ546179-B-CZE	87.8	92.3	93.1	85.9	87.7	92.6	95.3	92	96.9	91
HF968639-B-ESP	87.8	92.3	93.1	85.5	86.9	91.7	95.3	91.6	96.9	92.6
JQ647458-W-PRC	87.7	91.9	93.1	83.5	86.9	90.8	93.7	90.6	95.3	92.7
HF968646-B-AUT	87.5	91.5	91.9	85.8	87.7	92.3	94.6	91.8	96.1	91.8
JQ647508-W-PRC	87.4	90.2	89.5	84	86.9	91.2	93.7	89.7	96.5	92.7
AJ783960-B-TUR	87.3	91.4	93.1	85.5	86.9	92.9	96.1	91.9	97.7	90.2
HF968635-W-GBR	86.1	89.4	84.4	84.4	87.3	91.5	95	90	96.1	73.5
KM079154-W-POL	86.1	88.9	84.4	83.6	86.9	91.9	94.6	90.2	96.5	73
JN791095-W-IRI	86	90.2	87	83.9	87.3	90.8	93.7	89.6	96.5	74.2
AJ311031-W-SWE	85.9	89.8	84.4	84.2	86.9	90.8	94.2	89.5	96.5	74.5
HF968637-W-FRA	85.9	89.3	84.4	84	87.3	91.5	94.2	89.7	96.1	74
AM296023-W-DEU	85.9	89.3	84.4	83.7	86.9	91.2	93.7	89.6	95.7	74
JQ647467-W-HUN	85.7	88.9	83.1	84	86.9	90.9	93.7	89.5	96.5	72.4
EF536886-W-PRC	85.5	88.1	84.4	83.9	86.9	90.5	94.6	89.3	96.5	72.8
JQ647501-W-PRC	85.5	88.1	84.4	83.5	86.9	90.8	94.6	89.6	96.5	73.3
FJ546189-W-CZE	85.4	88.9	84.4	83.4	86.4	90.9	94.2	89.4	96.1	73
KJ536142-W-PRC	85.2	88.1	84.4	83.6	86.9	90.2	94.2	89	96.5	72.9
EU541489-W-PRC	85.2	88.1	84.4	83.6	86.9	90.2	94.2	89	96.5	72.9

(KJ536142 and EU541489) and Iran (JN791096) have been isolated from barley. This may indicate that some wheat and barley strains of WDV naturally infect both host plants.

Infectivity of the cloned genome of WDV-Bar[IRI]

To test the infectivity and fulfill the Koch's postulates for WDV-Bar[IRI] from barley, a partial dimeric clone (pBin20-1.4WDV) was constructed. Wheat and barley plants agroinoculated with this clone showed yellowing and dwarfing symptoms at 21 days post inoculation (dpi) (Fig. 3). Systemic infection and accumulation of the virus in the inoculated plants were confirmed by PCR. A representative PCR result is shown in Fig. 4. The virus was detected in 50.3% and 43% of inoculated barley and wheat plants, respectively (Table 2).

Discussion

The diversity and wide occurrence of WDV in wheat and barley fields causes a big challenge for breeders to produce plants with resistant or tolerant traits (Benkovics *et al.*, 2010). Identification and characterization of more strains or variants of the virus may shed further light on its taxonomy, spread, host specificity and the role of this virus in yellowing and dwarfing epidemics. In this study, a new variant of WDV from barley was isolated, sequenced and characterized. Moreover, the infectious clone of this isolate was prepared to test infectivity in both barley and wheat plants.

The full genome size of the isolated WDV in this study, shares 97.6 % sequence identity to a barley strain from Iran. Observing the rules by the International Committee on Taxonomy of Viruses (ICTV) for the genus *Mastrevirus* (Fauquet *et al.*, 2008) and the update on the mastrevirus taxonomy

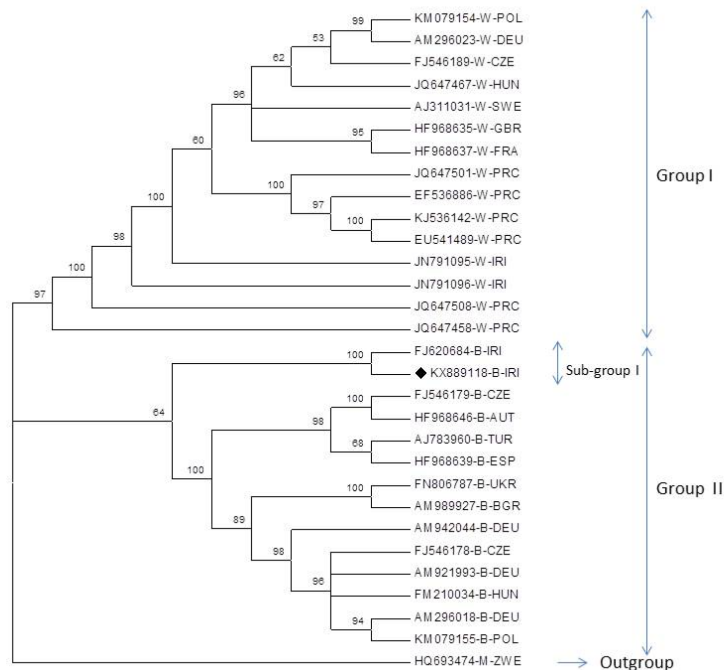

Fig. 2. Phylogenetic tree obtained from the alignment of nucleotide sequences of 29 barley and wheat isolates of WDV that show their relationship. B is for the barley strain and W for the wheat strain of WDV. The barley isolate from this study (KX889118) is indicated with a tetra angle in a sub-group with another barley isolates of WDV. A maize streak virus (HQ693474) was used as for outgroup. Table S1 provides the details of each sequence.

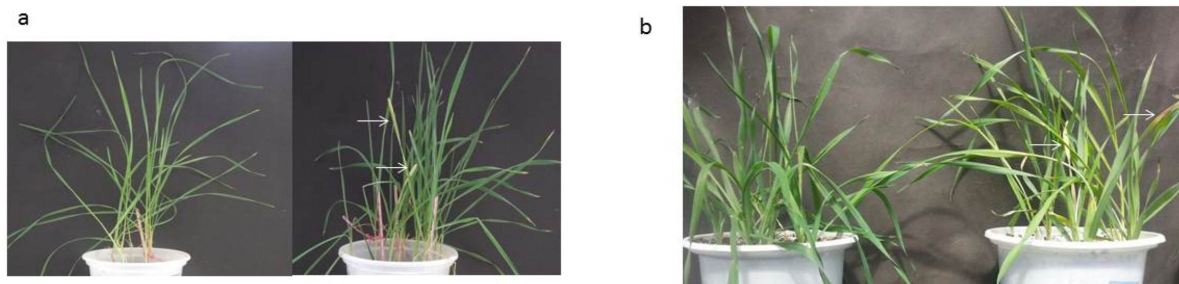

Fig. 3. The yellowing symptom in wheat (A) and barley (B) plants inoculated with WDV-Bar[IRI]. The left pot contains healthy plants. White arrows show yellow leaves in infected plants. Plants were photographed at 21 dpi.

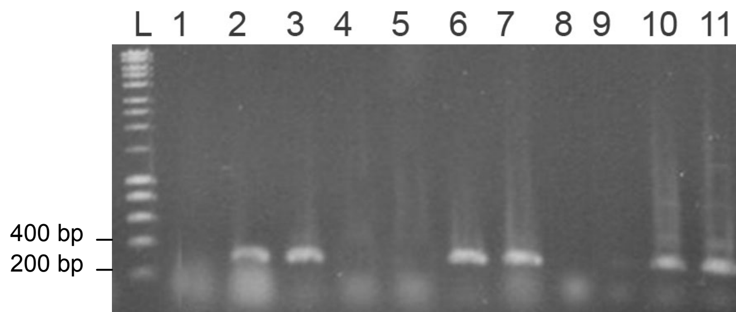

Fig. 4. A representative electrophoretic patterns of PCR products from inoculated wheat plants using WDV specific primers, WDV1F and WDV1R. Lane 1 and 2 are healthy and positive DNA control (pBSK-1.0WDV), respectively. Lanes 3 to 11 show WDV accumulation in the inoculated plants.

Table 2. Infection rate of WDV-B [IR] infectious clone in tested barley and wheat plants

	Host	No. of inoculated plants	No. of infected plants	Infection rate (%)
Experiment 1	Wheat	20	9	45
	Barley	16	9	56.25
Experiment 2	Wheat	12	5	41
	Barley	18	8	44.4
Experiment 3	Wheat	25	5	20
	Barley	25	4	16

(Muhire *et al.*, 2013), this WDV isolate which has more than 95 % sequence identity with other barley isolates of WDV was considered as a new variant of the virus, WDV-Bar[IRI].

The LIR region of the isolated WDV-Bar[IRI] shows the highest variation as compared to the nucleotide sequences from the coding regions. Similarly, the largest variation has also been reported in LIR in wheat and barley isolates of WDV (Köklü *et al.*, 2007; Kvarnheden *et al.*, 2002) which contains Rep binding site. This may reflect the conserved role for the coding genes by WDV strains.

Phylogenetic analysis shows that the isolated WDV from barley was grouped with barley strains of WDV from European countries. Nevertheless, this isolate was close to another barley isolate from Iran in a distinct sub-group. Analyzing more WDV isolates showed that all strains of WDV-Barley from Iran have been grouped together in a separate cluster (Ghodum Parizpour *et al.* 2016). This indicates the presence of unique barley isolates in this region in which barley and wheat have been grown for many centuries (Harlan, 1971). Results of other phylogenetic studies show that WDV isolates were divided into two main groups, wheat and barley (Köklü *et al.*, 2007; Schubert *et al.*, 2007). In addition, a more recent phylogenetic analysis for 243 WDV isolates confirmed that WDV-Wheat and WDV-Barley are divided into two super-clades (Ghodum Parizpour *et al.* 2016). Therefore, host plants play an important role in grouping the stains of this virus. They have suggested considering WDV and Barley dwarf virus as separate species. Similarly, a host-dependent grouping of mastreviruses infecting barley, wheat, and oat has been shown by phylogenetic analysis of their complete nucleotide sequences (Schubert *et al.*, 2007). However, there are examples that barley strains of WDV have been isolated from wheat plants (Wu *et al.*, 2015; Ghodum Parizpour *et al.* 2016). In addition, a barley strain was shown to infect wheat plants at a low rate, 0.4 %, using the insect vector (Wu *et al.* 2007). Therefore, WDV isolates may not grouped strictly according to their host plants. Using the infectious clone of WDV-Bar[IRI], we provide evidence that supports infection of barley strain in both wheat and barley plants. Given the high similarity of nucleic acids (78-86%) especially in the coding regions of barley and wheat strains of WDV (Table 1) (Kvarnheden *et al.*, 2002; Lotfipour *et al.*, 2013; Schubert *et al.*, 2007), this strains may infect wheat plants naturally. Finally, using insect vector for transmission of this strain will shed a light on virus-insect interaction and infectivity rate of the virus in host plants.

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Conflict of Interest

Both authors declare that they have no conflict of interest. Authors of this manuscript had no financial, commercial, political or personal interest that could inappropriately influence the contents of this manuscript. Authors have full control of all primary data and agree to allow the journal to review the data if requested.

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