

The Nucleotide Sequence of Two Hungarian Isolates of *Wheat Dwarf Virus*

I. TÓBIÁS¹, B. KISS¹ and L. PALKOVICS²

¹Plant Protection Institute, Hungarian Academy of Sciences,
H-1525 Budapest, P.O. Box 102, Hungary

²Department of Plant Pathology, Corvinus University of Budapest,
H-1118 Budapest, Ménesi út 44, Hungary

(Received: 15 December 2005; accepted: 20 January 2006)

Two wheat-infecting isolates of WDV – WDV-B and WDV-F – were collected in the field of Martonvásár and Nagykovácsi. The complete genomes were amplified by PCR, cloned into pBKS+ plasmid and sequenced. The nucleotide divergence in the total genome of the five isolates – WDV-Fra, WDV-Cz, WDV-Swe, WDV-B and WDV-F – originating from different part of Europe were found to be 0.44 – 1.69%. The four genes – MP, CP, RepA and Rep – and two non-coding region – LIR and SIR – were compared and a phylogenetic tree was constructed.

Keywords: *Wheat dwarf virus*, wheat-infecting isolates, sequence analysis, phylogenetic tree.

Wheat dwarf virus (WDV) was first described by Vacke (1961) in the former Czechoslovakia and later it has been found in Sweden (Lindsten et al., 1970), Bulgaria (Stephanov and Dimov, 1981), Hungary (Bisztray et al., 1989), France (Lindsten and Lindsten, 1993) and Germany (Huth, 2000). Wheat dwarf virus causes a severe disease of cereals, mainly winter barley and wheat. Affected plants show a yellowing of leaves, stunting, reduced root growth, delayed heading, and a reduction in yield. WDV is transmitted by the leafhopper *Psammotettix alienus* Dahlb. (Vacke, 1961), therefore the occurrence of diseased plants in the field depends on this vector. The incidence of WDV in crops differs greatly from year to year and between fields. Several important factors of the epidemiology of WDV are still poorly understood, such as alternative hosts in the field and virus variability. Two different forms of WDV exist: a wheat-adapted form (WDV wheat strain) and a barley-adapted form (WDV barley strain) (Lindsten and Vacke, 1991; Bendahmane et al., 1995).

Wheat dwarf virus belongs to genus *Mastrevirus* (family *Geminiviridae*) infects monocotyledonous plants. *Mastreviruses* have a monopartite single-stranded genome of circular DNA and the genome encodes four different proteins: movement protein (MP), coat protein (CP) and two replication-associated proteins (Rep and RepA) (Gutierrez, 1999). The presence of an intron in the rep gene makes it possible for WDV to produce the two different forms of the replication protein. The non-coding long intergenic region (LIR)

and short intergenic region (SIR) contain sequence elements necessary for viral replication and transcription. The complete genome is known for three wheat strains of WDV and a part of the genome has been determined of many WDV isolates (Kvarnheden et al., 2002).

The aim of this study was to characterise the strains of WDV occurring in Hungary and compare with the previously sequenced isolates from France (Accession number X82104), Sweden (Accession number: AJ311031) and Czechoslovakia (Accession number: X02869).

Materials and Methods

Virus Isolates

Leafhoppers (*Psammotettix alienus* Dahlb.) were collected in wheat field of near Martonvásár (Hungary GPS coordinates N: 47° 19' 49" E: 18° 46' 49") and Nagykovácsi (N 47° 32' 53" E 18° 55' 59") in summer 2003. The plants showed yellowing of leaves and reduced growth. The collected leafhoppers were placed on healthy wheat seedlings (10 individuals/10 plants in one plot) in insect-proof isolation net. Six weeks later the plants were tested for WDV with ELISA using WDV kit (Bio-Rad No. 31202).

Cloning and sequence analysis of the WDV isolates

Leaves were collected from WDV-positive plants. Total nucleic acid was extracted from 100 milligrams of wheat leaves using the method of White and Kaper (1989). Ethanol precipitated nucleic acid was redissolved in 40 µl of sterile water, 3 µl was used for polymerase chain reaction (PCR) in 50 µl volume. Primers (WDV-reverse 2313–2296 5'-CATAAGCTTCGT-GCTTCCATC-3' and WDV-forward 2308–2326 5'-TAAAAGCTTGTCTGCACGAGA-3') were designed to amplify the complete genome of WDV. The reaction mixture contained 20 pmol of primers, 200 µmol of each dNTP, PCR reaction buffer and 2.5 U Taq polymerase (Fermentas). Denaturation at 94 °C for 4 minutes was followed by 40 cycles of 20 seconds at 94 °C denaturation, 30 seconds at 60 °C annealing, 3 minutes and 30 seconds at 72 °C extension, with a final extension step at 72 °C for 10 minutes. The amplified DNA fragments were separated in 1% agarose gel and extracted by DNA purification kit (Fermentas). The PCR products were digested with *Hind* III and cloned into digested pBKS+ plasmid (Stratagene). The recombinant plasmids were transformed into *Escherichia coli* DH5α (Sambrook et al. 1989).

Clones containing inserts with the expected size of 2.7 kb were sequenced with DyeDeoxyTerminator Kit (Applied Biosystems, Foster, CA, USA) using reverse, universal (–20) and internal primers. Sequence analysis was performed using University of Wisconsin Genetics Computer Groups (GCG) sequence analysis software package version 9.1. Multiple sequence alignments were generated using PILEUP of GCG package. CLUSTALW was used to create an input file for PHYLIP. The tree was displayed using DRAWTREE program.

Results

Virus isolates

The collected leafhoppers produced WDV infection on wheat plants confirmed by ELISA tests. The WDV originated from Martonvásár was designated as WDV-B, and virus originated from Nagykovácsi as WDV-F. Both WDV isolates were maintained on wheat plants and leafhoppers were transferred every 6–8 weeks to healthy plants kept in insect-proof growth chamber.

The WDV-B and WDV-F isolates infect wheat (*Triticum aestivum*) and oat (*Avena sativa*) but barley (*Hordeum vulgare*) plants were not infected.

Cloning of the WDV and sequence analysis

The complete genomes were amplified by PCR, separated in 1% agarose gel (Fig. 1), purified, digested by *Hind* III and cloned into pBluescript KS+ plasmid.

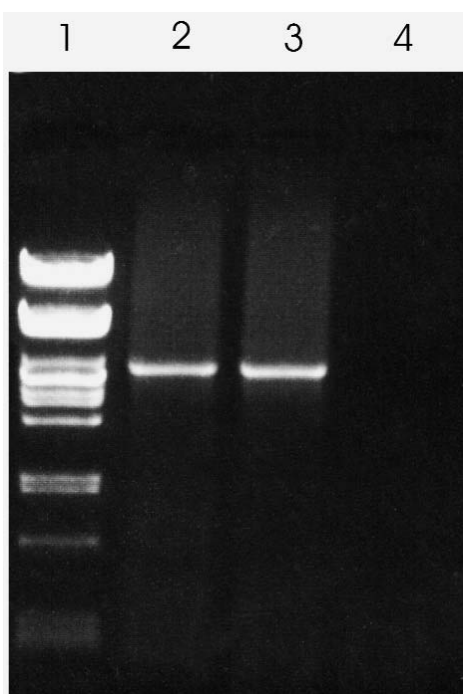


Fig. 1. Separation of amplified PCR products of WDV-B and WDV-F isolates on a 1% agarose gel stained with ethidium bromide (Lane 1: DNA length marker *Pst* I digested λ DNA, Lane 2: WDV-B isolate, Lane 3: WDV-F isolate, Lane 4: negative control)

The sequenced genome of WDV-B and WDV-F were exactly the same size – 2750 nucleotides – as that of previously sequenced WDV isolates. The nucleotide sequences were deposited into GenBank (Accession numbers: WDV-B: AM040732 and WDV-F: AM040733).

The genome contained the four genes (MP, CP, repA, rep) as well as the LIR and SIR like all mastreviruses. The nucleotide sequence differences of the complete genome among the wheat-infecting WDV isolates were between 0.44% and 1.69% (*Table 1*).

Table 1

The sequence homology of the complete genome of wheat-infecting WDV isolates
Abbreviation and accession numbers: WDV-B: AM040732, WDV-F: AM040733, WDV- Swe: AJ311031, WDV-Cz: X02869, WDV-Fra: X82104

	WDV-F	WDV-Swe	WDV-Cz	WDV-Fra
WDV-B	99.56	98.72	98.31	98.36
WDV-F		98.65	98.31	98.50
WDV-Swe			98.76	98.76
WDV-Cz				98.72

Hungarian WDV isolates (WDV-B and WDV-F) were very similar (99.56% homology) to each other, but differed from previously characterized Czech (WDV-Cz), France (WDV-Fra) and Swedish (WDV-Swe) isolates by 1.24–1.69%. However all wheat-infecting isolates were found to be genetically very similar.

The phylogenetic tree (*Fig. 2*) shows close relationship of the two Hungarian WDV isolates. The WDV isolates from France and Czechoslovakia are on the other side of the tree and Swedish isolates is in intermediate position.

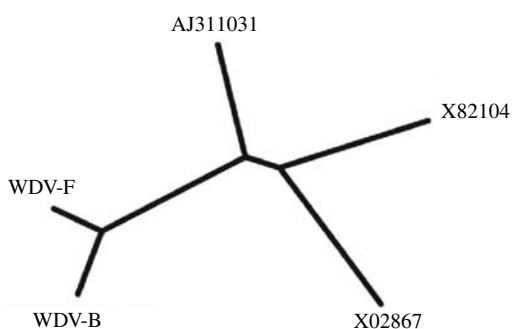


Fig. 2. Phylogenetic tree of WDV isolates (Abbreviation and accession numbers see in *Table 1*)

The four conserved coding regions – V1 (movement protein, MP), V2 (coat protein, CP), C1 (replication protein A, RepA) and C2 (replication protein, Rep) – were found to be less variable. The movement protein of WDV-B, WDV-F and WDV-Swe isolates are completely identical and show 98.9% homology with WDV-Cz and WDV-Fra isolates (*Table 2*).

The coat protein of both Hungarian WDV isolates are identical and show low level of variation (0.4–0.8%) comparing to other WDV isolates (*Table 2*), but differ more from Swedish and France than from the Czech WDV isolates.

Table 2

Sequence homology of V1 (movement protein) and V2 (coat protein) proteins
(above the diagonal V1 protein and below the diagonal V2 protein)

	WDV-B	WDV-F	WDV-Swe	WDV-Cz	WDV-Fra
WDV-B		100	100	98.9	98.9
WDV-F	100		100	98.9	98.9
WDV-Swe	99.2	99.2		98.9	98.9
WDV-Cz	99.6	99.6	99.6		98.9
WDV-Fra	99.2	99.2	99.2	99.6	

Interestingly the replication associated proteins (C1 and C2 proteins) of WDV-F, WDV-Swe and WDV-Cz, WDV-Fra are identical, respectively. These isolates show low difference (0.4–1.0%) with WDV-B and to each other (*Table 3*). The replication proteins of mastreviruses are multifunctional proteins containing several motifs and domains (Palmer and Rybicki, 1998; Gutierrez, 1999) and because of their importance during the virus life cycle they show high homology.

Table 3

Sequence homology of C1 (RepA) and C2 (Rep) proteins
(above the diagonal C1, below the diagonal C2 protein)

	WDV-B	WDV-F	WDV-Swe	WDV-Cz	WDV-Fra
WDV-B		99.6	99.6	99.0	99.0
WDV-F	99.5		100	99.6	99.6
WDV-Swe	99.5	100		99.6	99.6
WDV-Cz	99.0	99.5	99.5		100
WDV-Fra	99.0	99.5	99.5	100	

The noncoding regions – small intergenic region, SIR and large intergenic region, LIR – show higher level of sequence divergence, LIR is more variable than SIR (*Table 4*).

Table 4

Sequence homology of non-coding regions
[above the diagonal SIR (small intergenic region), below the diagonal LIR (large intergenic region)]

	WDV-B	WDV-F	WDV-Swe	WDV-Cz	WDV-Fra
WDV-B		99.4	99.6	98.2	98.2
WDV-F	98.8		97.0	97.6	97.6
WDV-Swe	97.6	97.1		99.4	99.4
WDV-Cz	96.6	96.3	97.1		100
WDV-Fra	96.0	96.7	96.6	97.5	

The sequence divergence in LIR was between 1.2% and 4%, while in SIR it was from 0.0% to 3%. The noncoding regions contain several putative regulatory regions, like

LIR region for binding of RepA and/or Rep proteins, a TATA-box for directing expression of Rep, and putative regulatory GC-boxes involved in the regulation of the viral-sense promoter (Hughes et al., 1993; Castellano et al., 1999; Missich et al., 2000). The relatively higher level of diversity, detected in non-coding regions, suggests that certain changes can be tolerated.

Acknowledgements

László Palkovics and Balázs Kiss were supported by the Bolyai János fellowship of the Hungarian Academy of Sciences. This work was supported by OTKA (grant K 61644).

Literature

- Bendahmane, M., Schalk, H.-J. and Gronenborn, B. (1995): Identification and characterization of wheat dwarf virus from France using a rapid method for geminivirus DNA preparation. *Phytopathology*, 85, 1449–1455.
- Bisztray, Gy., Gáborjányi, R. and Vacke, J. (1989): Isolation and characterisation of wheat dwarf virus found for the first time in Hungary. *J. Plant Dis. Protect.* 96, 449–454.
- Castellano, M. M., Sanz-Burgos, A. P. and Gutierrez, C. (1999): Initiation of DNA replication in an eukaryotic rolling-circle replicon: Identification of multiple DNA-protein complexes at the geminivirus origin. *J. Mol. Biol.* 290: 639–652.
- Gutierrez, C. (1999): Geminivirus DNA replication. *Cell Mol. Life Sci*, 56, 313–329.
- Huth, W. (2000): Viruses of gramineae in Germany – a short overview. *J. Plant. Dis. Protect.* 107, 406–414.
- Hughes, F. L., Rybicki, E. P. and Kirby, R. (1993): Complete nucleotide sequence of sugarcane streak Monogeminivirus. *Arch. Virol.* 132, 171–182.
- Kvarnheden, A., Lindblad, M., Lindsten, K. and Valkonen, J. P. T. (2002): Genetic diversity of *Wheat dwarf virus*. *Arch. Virol.*, 147, 205–216.
- Lindsten, K. and Lindsten, B. (1993): Occurrence and transmission of Wheat dwarf virus WDV in France. In: *Proceedings of Third International Conference on Pest in Agriculture*. Montpellier, France, 7–9 December 1993, pp. 41–48.
- Lindsten, K. and Vacke, J. (1991): A possible barley adapted strain of Wheat dwarf virus (WDV). *Acta Phytopath. Entomol. Hung.* 26, 175–180.
- Lindsten, K., Vacke, J. and Gerhardson, B. (1970): A preliminary report on three cereal virus diseases new to Sweden spread by *Macrostelus* and *Psammotettix* leafhoppers. *Nat. Swedish Inst. Plant Protec. Cent.* 14, 285–297.
- Missich, R., Ramirez-Parra, E. and Gutierrez, C. (2000): Relationship of oligomerization to DNA binding of wheat dwarf virus RepA and Rep proteins. *Virology* 273, 178–188.
- Palmer, K. E. and Rybicki, E. P. (1998): The molecular biology of mastreviruses. *Adv. Virus Res.* 50, 184–234.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989): *Molecular cloning. A Laboratory Manual*. 2nd ed. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.
- Stephanov, J. and Dimov, A. (1981): Bolestta vdjudjavanje po spenittsata Bolgaria. *Rasteniev Nauki* 18, 124–128.
- Vacke, J. (1961): Wheat dwarf virus. *Biol. Plant.* 3, 228–233.
- White, J. L. and Kaper, J. M. (1989): A simple method for detection of viral satellite RNAs in small tissue samples: *J. Virol. Meth.* 23, 83–94.