Investigation of Differences between Wheat and Barley Forms of *Wheat Dwarf Virus* and their Distribution in Host Plants

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Abstract

Wheat dwarf virus, a monogemini virus, infects several cereal species. Until now complete sequence data have been published only for wheat isolates. We cloned the complete DNA of 21 isolates from wheat, barley and *Lolium* spec. and compared the sequences with published data. Two types of the virus were found as previously described. Degree of entire nucleic acid homology between both isolates was in the range of 84%. The Large Intergenic Region showed most pronounced differences while the RepA gene was most conserved. No intermediate forms were found, though both isolates co-existed in the same hosts. Sequence data lead to the suggestion that they should be referred to as different viruses rather than strains of a virus.

Keywords: *Wheat dwarf virus*; isolates; sequence; hosts

INTRODUCTION

Wheat dwarf virus (WDV) is a member of the genus *Mastrevirus* within the family *Geminiviridae* which is characterized by the geminate morphology of the capsid and by circular ssDNA genomes. The geminivirus family has been divided into four genera mainly on the basis of virus vector species, host range and genome organization. Species of the genus *Mastrevirus* are transmitted by leafhoppers, have a monopartite genome, and generally infect monocotyledonous plants. To this date (see also http://www.ictvdb.iacr.ac.uk/ICTV/Genus1), 14 definitive members of the genus and two tentative species have been identified (FAUQUET *et al*. 2000).

WDV was first reported in *Triticum aestivum* from the former Czechoslovakia (VACKE 1961). Meanwhile WDV was described for several regions of Europe and North Africa (KVARNHEDEN *et al*. 2002). The phloem limited virus is transmitted by the leafhopper *Psammotettix alienus* (Dahlb.). Characteristic symptoms of the disease are yellowing/streaking and severe stunting.

Two forms of WDV are described: a wheat and a barley adapted form (LINDSTEN & VACKE 1991) which can be distinguished by host range and by means of sequence specific PCR-primers (COMMANDEUR & HUTH 1999). Sequences of primers were not published. Differentiation by means of polyclonal antisera appeared to be not possible (VACKE & CIBULKA 2000). According to LINDSTEN and VACKE (1991) barley adapted forms do not infect wheat while COMMANDEUR and HUTH (1999) reported that they do.

Until recently complete sequences have been published only for wheat isolates. The genome of the wheat forms encodes five different proteins: movement protein (MP; V1) and coat protein (CP, V2) on the virion strand and the RepA (C1) and Rep (C1:C2) proteins on the complementary strand as well as a Cx protein with unknown function. The replicase (Rep) is the product of splicing. In addition, all *Mastreviruses* have in common a large and

a short intergenic region designated as LIR and SIR, respectively (Figure 1).

MATERIAL AND METHODS

Plant material, extraction of DNA

Material from field grown barley (Kreptitz, Island of Ruegen, Germany) was collected and tested by DAS-ELISA for presence of *Barley yellow dwarf virus* (BYDV) and WDV. Though WDV was not detected in purified BYDV preparation, after cDNA synthesis and cloning a contamination with WDV was found. Sequences of the corresponding clones served as starting point for generation of WDV specific primers (A/D and J/E, Figure 1). Later on DNA of WDV was isolated from 50 mg of fresh plant material by three methods: the guanidine/alkaline extraction method (GTC-method; BENDAHMANE *et al*. 1995), a guanidine-isothiocyanate/phenol method (GITC-method; peq-Gold DNA-Pure; PeqLab) and a plant DNA extraction kit (Nucleo Spin Plant, Macherey-Nagel). Viral DNA was extracted and cloned using host plants from different regions of Germany: barley – Walbeck (Saxony-Anhalt, maintained in a climate chamber; Wal); Krostitz (Saxony, field sample; Kro), Aschersleben (Saxony-Anhalt, field sample; VF); Kreptitz (Mecklenburg-Vorpommern, field sample; Kre); wheat – Alsleben (Saxony-Anhalt, kept in a climate chamber [collected in 2002] and field isolate [collected 2001]; Als); not identified *Lolium* species-Aschersleben (field sample; LP).

PCR-reaction, cloning and sequencing

Sequences of primers are shown in Table 1, their location on genome in Figure 1. The overlapping A/D and J/E PCR-fragments covered the entire genome of WDV (Figure 1). By means of these non-specific primers it was possible to amplify DNA from all field samples of barley showing typical symptoms of WDV infection. As fragment A/D of isolate VF (RepA/partial Rep-fragments) was not clonable, primers F/A and D/G (Figure 1) were used and the resulting shorter fragments could be cloned. Primer set H/I was designed for amplification of full length product.

PCR-reaction was performed with Triplemaster-Polymerase (Eppendorf, a Taq-polymerase with proofreading activity) in a 50 μl-reaction mixture with 1× tuning buffer (supplied), 0.2 mM dNTPs, 5 μl DNA, 0.2 μM of each primer and 1 U enzyme. Cycling conditions were: 2 min 94°C, 8× (30 s 94°C, 30 s 62°C [lowering annealing temperature for 0.5°C in each cycle], 2 min 72°C), 20× (30 s 94°C, 30 s 58°C, 2 min 72°C), 8 min 72°C. Resulting bands were excised from agarose gel, purified (Invitec DNA gel purification kit) and cloned into pGEM-T (Promega). Two clones were chosen for sequencing on an ALF-Express sequencer (Amersham-Pharmacia, cycle sequencing kit).

Expression studies

The coat protein (CP) gene of a clone originating from isolate ASL-1 (ASL CP1), was expressed in *Escherichia coli*. For this purpose the CP fragment was reamplified with primers B and C (Figure 1) and cloned via NcoI/SalI into pET30a (Novagen) or pThioHis (Invitrogen). After induction with 1 mM IPTG at 30°C for 20 h cells were collected by centrifugation, resuspended in distilled water (in 2 ml from a 20 ml culture) and disrupted by ultrasonication. 5 μl of the sonicate were denatured with 5 μl 2× loading buffer and loaded onto an 8% acrylamide gel. After electrophoresis the proteins were blotted onto PVDF-membranes (Roth) and developed using a WDV-IgG (Fuchs, University Halle/Saale) or a His-MAb (Novagen) with an anti-rabbit or anti-mouse AP-conjugate (Bioreba), respectively. As substrate BCIP/NBT were used.

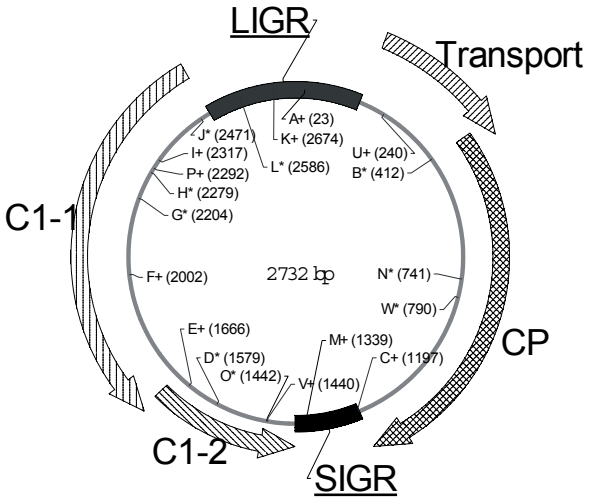

Figure 1. Gene map of the DNA of a typical barley form of WDV. Primer positions (compare to Table 1) are included (* clockwise, + counterclockwise direction of synthesis, respectively). Positions K-N correspond to positions Q-T for the wheat form, respectively

Table 1. Sequences of primers used for different amplification purposes

Primer designation	Sequence (5'→3')	Used for amplification of
A(3-100)	GCACTCACGAAAAGCCGAGTGCG	DNA from both forms
D(5-1700)	TGAGCCCCGACGAAGCCCTTCC	
E(3-1700)	GACCTATGACGTCAACGCCAAGTACAACATAA	
J(5-2600)	GGAACCTGGGTGCAGATGAAGAGGC	
B(CP5-NcoI)	ATCCATGGAAATGGTGACCAACAAGGACTCC	CP gene for cloning in expression plasmid
C(CP3-SalI)	ATGTCGACGTATTGAATCCCAATGGATTTGAAGTAAC	
Q(WE2700-3)	CTACGCTMCCGTGGGAACAC	DNA from wheat isolates
R(WE2600-5)	TCTGCACACTGGAAATGCAAT	
S(WE1340-3)	ATAAATGTAATATCTCAGGGTG	
T(WE720-5)	ATCGGTCCAGTCCGCCTC	
K(GE2700-3)	CGCTTTCGCGCCGAAAAGAC	DNA from barley isolates
L(GE2600-5)	AATTCACACCGATCGGCTCG	
M(GE1340-3)	AAAATTGTGTGATCTCGCCCC	
N(GE720-5)	CATAACCCCAATCAGGGTTTAT	
F(C1-Int3)	GGATAGACCATTCAAACGGAAAACG	shortened fragments of E/J
G(C1-Int5)	GACTGCAACCAAGTTCGTGATTAC	
O(5-1500)	TAGAGTACATGTAATCCGACTGTTC	
P(3-2300)	GCTTCCATCACCAATCCCAATGC	
H(FL-AS)	TGGTGATGGAAGCACGAAGCTTGTTTTG	full length DNA (HindIII underlined)
I(FL-S)	ACGTACTCGTGCAAAACAAGCTTCG	
U(WE240-3)	ATCGACATGGGCTACCACG	wheat forms, use with R
V(GE1440-3)	GGGTGAATCATTCTTCGCCTT	barley forms
W(GE790-5)	CTCCTCATGTATGTTCATAACC	

RESULTS

PCR-analysis of infected leaf material resulted in specific PCR bands independently from isolation procedure (Figure 2) though those from GITC-DNA have been sometimes only weak. Use of primer pair H/I designed to obtain full length sequence did not result in a PCR-band.

As the region containing the replicase gene was sometimes not clonable, shorter fragments have been amplified and cloned. Resulting sequences were combined to full length sequences.

On the basis of the alignment wheat and barley form specific primers were developed and used for amplification of the corresponding genomic regions. Clones LP**B**/LP**W** (*Lolium*, **b**arley and **w**heat isolate) and WeAls1-3 (barley, wheat isolate), representing full length sequences, resulted from the use of specific primers. In some cases fragments from specific bands were not clonable too. By means of the specific primers it was possible to demonstrate that wheat, barley and *Lolium* are infected by both forms of WDV.

Alignment of complete sequences and of separate genes/regions derived from them was performed by Clustal W/Neighbor-Joining analysis. Percentage of sequence homology for separate genes/regions is outlined in Table 2. Reliability of the results was checked by bootstrap analysis (trial number 1 000). A characteristic clusters is shown in Figure 2.

Two sets of primers were synthesized (U/R, V/W) to test whether a multiplex differentiating analysis is possible. Although the primers showed excellent specificity as confirmed by sequencing a combined detection of both WDV forms in one reaction failed (Figure 3).

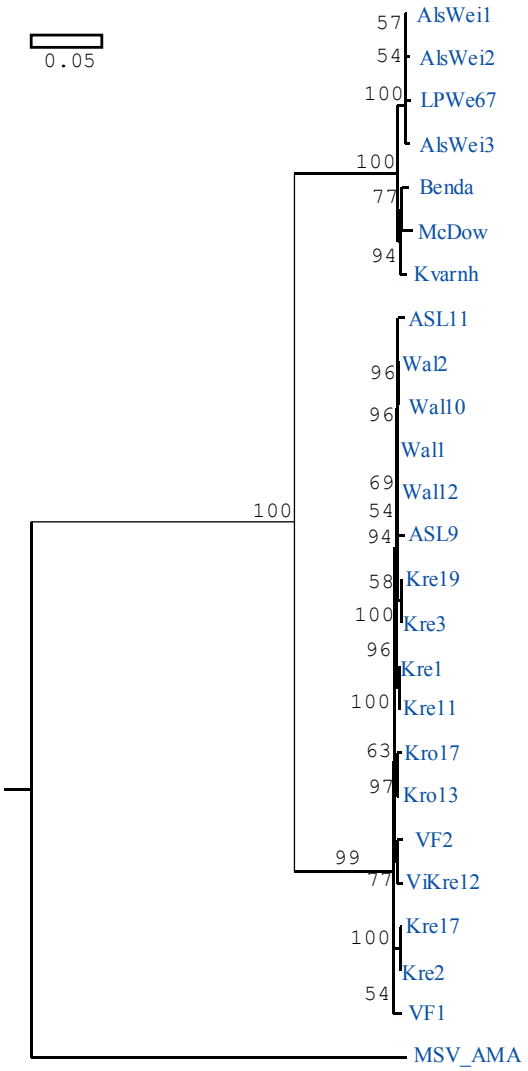

Figure 2. Phylogenetic analysis of complete WDV genome sequences of several barley and wheat forms: Bootstrap analysis (trial number 1 000). Neighbor-Joining and UPGMA analysis result in a similar cluster

Expression of CP gene

Polyclonal antisera, available for detection of WDV have some background reaction with healthy material which hinders detection of small amounts of the virus as shown for purified preparation. Production of new antisera is hard to achieve as only little amount of the virus can be purified from infected barley plants. For this reason we decided to express the CP gene in *E. coli*. Experiments gave two unexpected results: the CP was insoluble, even if fused to thioredoxin and attempts to solubilize the protein in urea or guanidine-HCl led to destruction of recombinant CP (not shown). Thus, a purification of the recombinant protein by IMAC will not be possible.

DISCUSSION

Phylogenetic analysis indicated that both WDV forms are highly homologous but clearly different from each other. The best investigated virus of genus *Mastrevirus* is *Maize streak virus* (MSV). For it several sequence specific clusters with rather high sequence diversity were identified (WILLMENT *et al*. 2002). Thus, Tas- and VW-strains from grass showed only 79% sequence homology with typical maize isolates. In addition, it was demonstrated that between different isolates recombination may appear (MARTIN *et al*. 2001). In contrast to these findings the variability of both WDV forms is low (Table 2). No evidence was found for a recombination event between them, though several other shorter sequences have been investigated (results omitted). Both forms reveal another difference supporting this opinion. All investigated wheat forms own an open reading frame with unknown function named Cx. This ORF is completely missing in all investigated barley isolates.

Table 2. Nucleic-/amino acid homology among different genes/regions of the WDV genome

DNA-segment	% homology among			
	barley forms	wheat forms	barley/wheat forms	WDV/MSV
Complete genome	99/–	98/–	84/–	46/–
LIG	97/–	97/–	72/–	41/–
CP	98/96	98/98	82/86	43/30
Transport	99/98	99/99	85/81	41/32
SIG	97/–	98/–	83/–	47/–
RepA (5'-end)	98/98	99/99	89/91	46/42
RepA (3'-end)	99/97	99/98	89/93	59/53

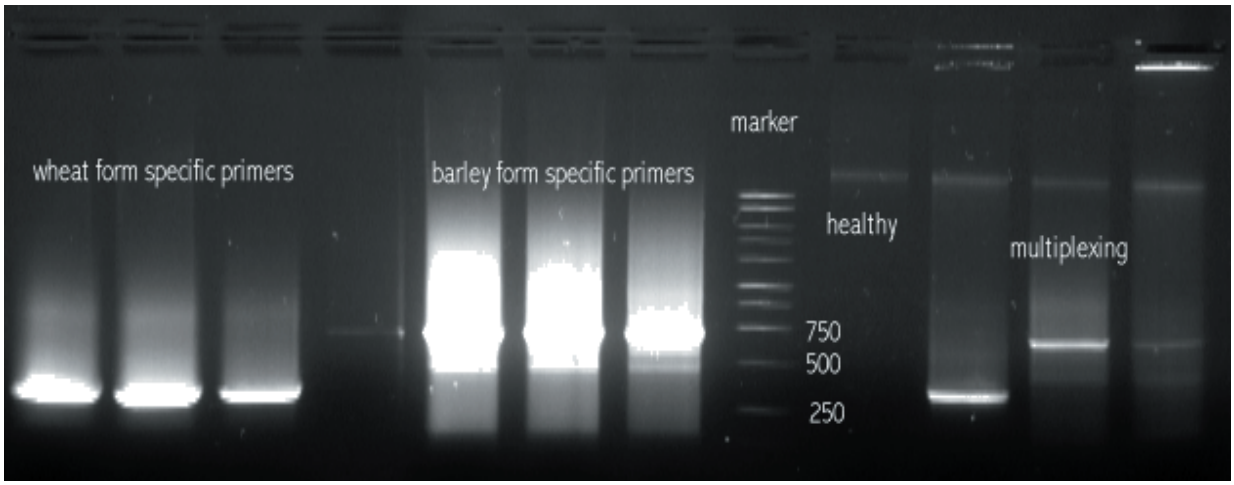

Figure 3. Detection of both forms of WDV in field material (barley) with primer sets U/R (wheat specific) and V/W (barley specific) in case of single use (left from marker) and multiplexing (right from marker)

WDV, wheat form:	**L**I**C**H**E**
WDV, barley form:	**L**M**C**H**E**
Digitaria streak virus:	**L**L**C**Q**E** (M23022)
Panicum streak virus:	**L**H**C**N**E** (X60168)
Maize streak virus:	**L**L**C**N**E** (e.g. AF239960)

None motif for: *Egyptian sugarcane streak virus* (AF039528, AF037752); *Chlorosis striate mosaic virus* (M20021), *Sugarcane streak virus* (M82918), *Miscanthus streak virus* (D00800)

Figure 4. Retinoblastoma-motif **L**x**C**x**E** of different *Mastreviruses* (accession number of sequences in brackets)

Wheat M_{102}SDASAPFIGPVRL…I_{226}SSTRGGVTGDSASTAFD…

Barley V_{102}SDSSCMFITPIRV.. V_{226}TCTRGGITGDSASISFE…

Figure 5. Alignment of deduced amino acid sequences of most diverse regions of the CP's of a wheat (clone WeALS2) and barley form (clone Wal1) of WDV, representative for all other isolates

The retinoblastoma-motif LxCxE of RepA (DURFEE *et al*. 2000) has a different structure for both forms as it seems to be characteristic for most *Mastreviruses* (Figure 4). Taken together these differences are an indication that one deals with two different viruses rather than with two forms of one virus.

The CP gene is especially interesting with respect of detection of the virus and differentiation of both forms by means of antisera. Homology analysis of nucleic and amino acid sequences of the CP clearly demonstrated that they fall into two independent clusters. Unfortunately, amino acid differences are dispersed over the entire sequence. Consequently, one can not expect that polyclonal antisera could differentiate between both forms. Nevertheless, two extended stretches exist which might enable the production of differentiating monoclonal antibodies utilizing synthetic peptides. They are located in the central as well as C-terminal part of the protein (Figure 5).

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