Identification and Characterization of Wheat Dwarf Virus from France Using a Rapid Method for Geminivirus DNA Preparation

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The nucleotide sequence of WDV-F has been deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number X82104.

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ABSTRACT

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Using molecular analyses, we identified wheat dwarf geminivirus (WDV), a leafhopper-transmitted geminivirus prevalent in northern and eastern Europe, as the causative agent of a recent wheat dwarf outbreak in France. A novel, simple, and rapid method for purification of circular DNA from small amounts of plant tissue was described and was applied to clone the genome of WDV-F. The cloned WDV-F DNA was shown to be infectious on wheat (*Triticum aestivum*) following agroinoculation, and progeny virus was transmitted by leafhoppers. The DNA sequence of the WDV-F genome consists of 2,750 bases, differs by only 1.3 and 1.4% from the WDV isolates from Sweden and Czechoslovakia, respectively, and represents a new isolate of WDV.

Additional keywords: DNA preparation, guanidine-thiocyanate, *Psammotettix alienus*, single-stranded DNA.

Wheat dwarf, a prevalent disease in northern and eastern Europe, is transmitted by the leafhopper *Psammotettix alienus* (29,44). The typical symptoms of leaf streaking, severe stunting, and reduced seed set are caused by wheat dwarf geminivirus (WDV) (28), whose host range includes wheat, barley, oats, and a wide variety of grasses (45). Geminiviruses are single-stranded DNA plant viruses with a unique double icosahedral capsid morphology (16,19,26). Geminiviruses are transmitted by different leafhopper species or by the whitefly *Bemisia tabaci*. All leafhopper-transmitted geminiviruses possess an undivided genome consisting of a single-stranded circular DNA (ss-DNA) molecule of about 2.7 to 3 kb; whereas, with the exception of the tomato yellow leaf curl viruses (TYLCVs) (13,22,35), most whitefly-transmitted geminiviruses have a bipartite genome comprised of two ss-DNA molecules of approximately 2.7 kb each. Leafhopper-transmitted geminiviruses infect both mono- and dicotyledonous plants (18). Geminiviruses have attracted considerable interest by plant molecular biologists with regard to both crop engineering via pathogen-derived resistance and virus-based vector development (11).

We describe a rapid and simple method to purify circular DNA from small amounts of plant tissue that we used to clone a variety of infectious geminivirus genomes. Here we report cloning of the infectious genome of a WDV isolate from France (WDV-F) using DNA prepared by this method. A comparison of its sequence with that of WDV isolates from northern and eastern Europe (30,46) and the significance of sequence conservation among geminiviruses of monocotyledonous, as opposed to those of dicotyledonous, plants is discussed.

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MATERIALS AND METHODS

Detection using squash blot. Plants (*Triticum aestivum* L.) showing symptoms like those induced by WDV infection were collected in 1989 from wheat fields of the Yonne and Cher regions in central France (H. Lapierre, INRA-Versailles, France). Infected leaves were squashed onto nylon membranes (Hybond N; Amersham Corp., Arlington Heights, IL) and hybridized as described by Navot et al., (34) using [α^{32}P]dCTP-labeled DNA of a WDV isolate from Sweden (WDV-S) (32) as a probe. Washing was under stringent conditions (twice in 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS] at 65°C). Standard molecular biology techniques were according to Sambrook et al. (41).

The guanidinium/alkaline DNA extraction method (GA-method). A slice (about 50 mg) of geminivirus-infected plant tissue was frozen in liquid nitrogen and ground to a fine powder in an Eppendorf tube, and 150 µl of guanidinium extraction solution (4 M guanidine thiocyanate [Aldrich Chemical Co., Milwaukee, WI, or Fluka Chemical Corp., Ronkonkoma, NY], 25 mM sodium-acetate [pH 5.2], 0.5% laurylsarcosine, and 0.1% dithiothreitol) were added (7). After 10 min at room temperature, 300 µl of alkaline lysis mix (0.2 N NaOH, 1% SDS) were added, followed by a further 10 min of incubation at room temperature. After the addition of 225 µl of 3 M potassium acetate (pH 4.8), the solution was chilled on ice (10 min) and cleared by centrifugation for 15 min in the cold (benchtop centrifuge, 17,000 × *g*). The supernatant was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the DNA was precipitated by the addition of 2.5 volumes of ethanol in the presence of 5 µg of yeast tRNA. The pellet was washed twice with 70% ethanol and redissolved in 50 µl of Tris-EDTA buffer(10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8]).

Analysis and cloning of the DNA. Aliquots of DNA purified by the GA-method were subjected to digestion with restriction enzymes and analyzed by Southern blot hybridization using a WDV-S probe. Linear DNA generated by *Sst*I digestion was used for cloning in *Sst*I-linearized plasmid pBluescript II KS$^+$ (Stratagene Inc., La Jolla, CA). Sequencing was done by the dideoxy-chain-termination method of Sanger et al. (42) using T7-DNA polymerase (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Construction of a partially redundant clone of WDV-F. Because the WDV was not mechanically transmitted, partially redundant clones of the WDV-F genome were constructed in plasmid pUC118 and in the binary plant transformation vector pBin19. A *Sst*I-*Bst*EII fragment of about 2 kbp was introduced between the *Sst*I and *Sma*I sites of the pUC118 polylinker, and then a full-length *Sst*I fragment of WDV-F was inserted in the *Sst*I site to yield a partially redundant WDV genome (pWF1.8 in pUC118 and pBWF1.8 in pBin19, respectively). The plasmids were propagated in the *Escherichia coli* strain DH5α. pBWF1.8 was introduced into *Agrobacterium tumefaciens* strain C58 harboring the Ti-plasmid pGV3850 (47) following the protocol of Höfgen and Willmitzer (20). Transformants were selected on yeast extract broth (YEB) plates containing kanamycin (100 µg/ml) and carbenicillin or ampicillin (100 µg/ml). The presence of pBWF1.8 in *A. tumefaciens* was verified by alkaline minipreparation of plasmid DNA adapted to *Agrobacterium* (14) and Southern blot hybridization analysis using full-length WDV-F DNA as a probe.

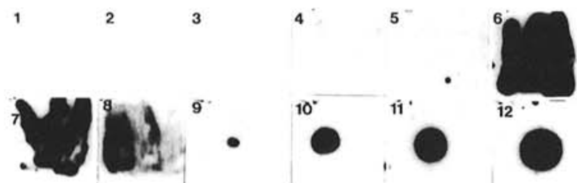

Fig. 1. Diagnosis of wheat dwarf geminivirus (WDV) in diseased wheat plants by squash blot. Leaves were squashed on a nylon membrane, and ^{32}P-labeled DNA of WDV-S (from Sweden) was used as probe. 1 and 2: leaves of healthy control plants; 3: *Digitaria setigera* infected with Digitaria streak virus; 4 through 8: leaves of field samples (*Triticum aestivum*), three of which (6 to 8) show strong hybridization. 9 through 12: 0.1, 1, 10, and 100 ng of denatured DNA of WDV-S.

Transfection of suspension cell protoplasts. Preparation and transfection of *T. monococcum* L. suspension cell protoplasts were carried out as described previously (32). Two million protoplasts were transfected with 20 µg of pWF1.8 DNA. After transfection, the protoplasts were incubated at 26°C in the dark. Protoplast-derived cells were harvested at 3 and 7 days after transfection, and total DNA was purified and analyzed by agarose gel electrophoresis followed by Southern blotting.

Agroinoculation and analysis of plants infected with the WDV-F genome. Agrobacteria harboring pBWF1.8 and the Ti-

Fig. 2. Forms of wheat dwarf geminivirus from France (WDV-F) DNA in naturally infected wheat plants. The DNA was purified from infected wheat leaves by the GA-method. **A,** Separation of purified WDV DNA on a 1% agarose gel stained with ethidium bromide. **B,** Autoradiograph of the corresponding Southern blot. DNA was blotted onto a nylon membrane (Hybond N, Amersham), and probed with WDV-S (from Sweden) DNA labeled with [α^{32}P]-dCTP. Lane 1: undigested DNA. Single-stranded (ss-) and covalently closed circular (ccc-) DNA forms are visible in the ethidium bromide stained gel. Lin = linear DNA of WDV. Lane 2: DNA treated with S1-nuclease. Lane 3: DNA digested with *Sst*I endunuclease. Lane 4: DNA from a healthy plant. Lane 5: DNA from a healthy plant, digested with *Sst*I endonuclease. Lane M: DNA length marker, as indicated to the right (kilobases).

plasmid pTi3850 were used for agroinoculation by vacuum infiltration (2). Wheat seedlings (*T. aestivum* cv. Briscard) of about 10 cm in size with two to three leaves were incised at a position near the meristem, completely submerged in a suspension of a five times concentrated (OD_{600} = 30) stationary culture of *Agrobacteria*. Between five and 15 bursts of a strong vacuum were applied and the seedlings were planted in soil. Plant survival under these conditions was about 99%.

RESULTS

Detection. Field-infected wheat leaves analyzed by squash blot hybridization tests showed a strong signal with the WDV-S probe after stringent washing. Specificity of the probe for WDV was demonstrated by lack of hybridization with leaf squashes of *Digitaria setigera* infected with Digitaria streak virus (DSV) (12) and healthy wheat leaves (Fig. 1).

The analysis of DNA prepared by the GA-method is illustrated in Figure 2. Panel A displays the separation on a 1% agarose gel stained by ethidium bromide; note the absence of any contaminating high-molecular-weight plant DNA in the samples. Panel B shows a Southern blot analysis of the same gel. Lane 1 (undigested DNA) shows the typical forms of geminivirus DNA: ss-DNA and the replicative covalently closed circular DNA (ccc-DNA). Treatment of the DNA with S1-nuclease eliminated the ss-DNA and converted the ccc-DNA into a linear form (Fig. 2, lane 2). Digestion by restriction endonuclease *Sst*I left the ss-DNA intact and converted the ccc-DNA into a single linear fragment of about 2.8 kb in size. Restriction by endonucleases *Bam*HI and *Sph*I did not linearize the circular double-stranded viral DNA forms (not shown).

DNA linearized by *Sst*I were cloned in plasmid pBluescript KS^+ (Stratagene). Using GA-prepared DNA, three out of 12 plasmids analyzed were recombinants containing a full-length copy of WDV-F (pWDV-F clone). The complete nucleotide sequence of WDV-F (2,750 bases) was determined using a nested deletion strategy similar to the one described by Kheyr-Pour et al. (22); gaps were bridged by appropriate specific sequencing primers. The sequence of WDV-F was deposited under accession number X82104 in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases. Figure 3 illustrates the genome organization of WDV-F. Open reading frame (ORF) C1-2 did not begin with an AUG codon, as was the case in all geminiviruses of monocotyledonous plants except maize streak virus (MSV).

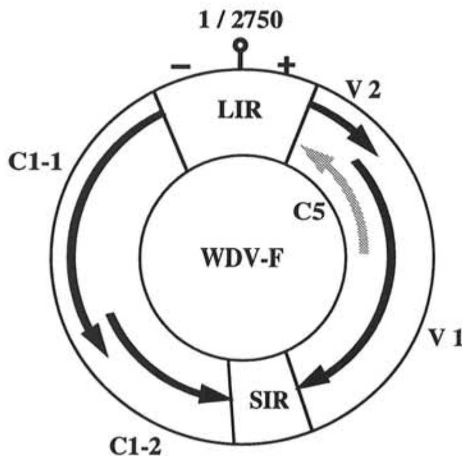

Fig. 3. Genome organization of wheat dwarf geminivirus from France (WDV-F). The four open reading frames (ORF) encoding identified proteins are shown as black arrows: V1 (capsid protein) and V2 on the plus strand, C1-1 and C1-2 (Rep protein) on the complementary strand. A fifth ORF (C5) whose significance remains to be determined is displayed in grey. The large intergenic region (LIR) of 403 bp containing the conserved potential stem-loop structure (l) with the nonanucleotide TAATATT[1]AC, and the small intergenic region (SIR) of 178 bp are indicated. The positions of the promoters of viral and complementary sense transcripts are marked by (+) and (–), respectively. Sequence numbering begins at the A (base 8 of the conserved nonamer) following the recommendation for geminivirus nomenclature issued at the 1st International Symposium on Geminiviruses, Almeria, Spain, in 1994.

Cloned WDV-F replicates in *T. monococcum* cells. A partially redundant clone of the WDV-F genome (pWF1.8) was used to transfect protoplasts of *T. monococcum* suspension culture cells. At 3- and 7-days posttransfection, total cellular DNA was prepared, fractionated on agarose gels, and analyzed by Southern blotting. Figure 4A shows a typical Southern blot of a WDV DNA replication in protoplast-derived cells. The replicative forms of WDV DNA, open-circular (oc-DNA), and covalently closed-circular molecules accumulated at 3 and 7 days. By contrast, the single-stranded DNA was detected only at 7 days posttransfection. Input DNA and de novo synthesized DNAs were distinguished by their difference in size (Fig. 4A). The amount of newly synthesized virus DNA increased from 3- to 7-days post-transfection (Fig. 4A, lanes 1 and 2). As a positive control for protoplast performance in the experiments, pW*neo*, a dimer of a recombinant WDV-S genome that carried the neomycin phosphotransferase gene (*npt*II) instead of the capsid protein gene (17,32), was used (Fig. 4A, lane 3). When digested with the endonuclease *Sst*I that cut the WDV-F and WDV-S*neo* DNA only once (Fig. 4C), the ccc- and oc-DNAs were converted to linear double-stranded DNA, whereas the single-stranded DNA was not

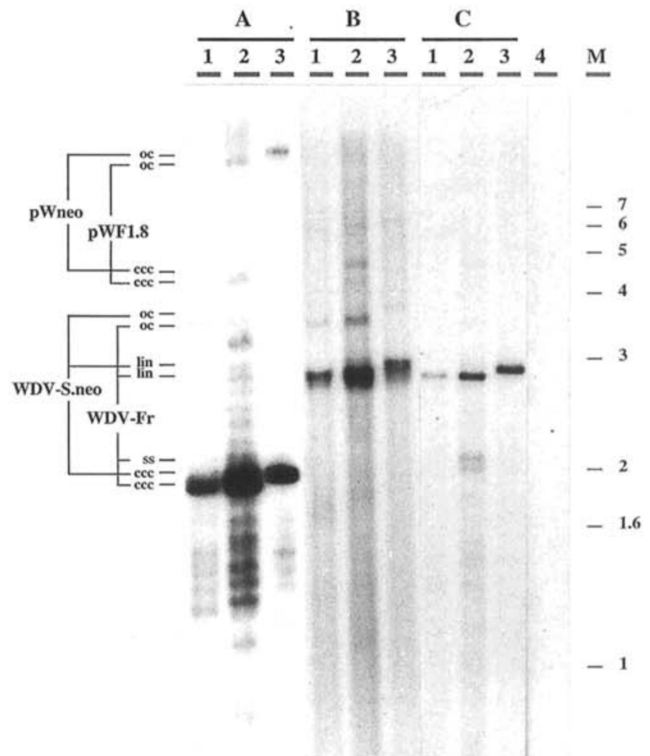

Fig. 4. Replication of wheat dwarf geminivirus from France (WDV-F) and WDV-S*neo* in protoplasts derived from the cell suspension culture of *Triticum monococcum.* Ten micrograms of total DNA from transfected cells were separated on a 1% agarose gel, blotted onto a nylon membrane (Hybond N, Amersham), and probed with WDV-F DNA labeled with $[\alpha^{32}P]$-dCTP. DNA was isolated from protoplast-derived cells transfected with pWF1.8 at 3 days (lane 1) and 7 days (lane 2), and from protoplast-derived cells transfected with pW*neo* at 5 days (lane 3). Lane 4 contains DNA from mock transfected protoplasts. Note that the recombinant WDV*neo* DNA is about 100 bp longer than wild-type WDV DNA. **A,** native viral DNA forms; **B,** DNA digested with S1-nuclease; **C,** DNA restricted with *Sst*I endonuclease. The positions of the open circular (oc-), covalently closed circular (ccc-) and single-stranded (ss-) forms of the respective DNAs are indicated. M: position and sizes (kilobases) of the fragments of the 1 kb DNA-ladder (Gibco BRL, Life Technologies, Gaithersburg, MD).

affected (Fig. 4C, lane 2 for WDV-F). Treatment with S1-nuclease (Fig. 4B) resulted in the disappearance of the single-stranded DNA (Fig. 4B, lane 2). The ccc-DNA form was converted to the linear form by the nicking activity of S1-nuclease at partially single-stranded regions of presumed secondary structures (Fig. 4B, lanes 1, 2, and 3). The DNAs running ahead of the bulk of the ccc-molecules probably represented topoisomers of ccc-DNA differing in their linking number.

Cloned WDV-F DNA infects wheat (*T. aestivum*). Six weeks after vacuum infiltration with *Agrobacterium* strain GV3850 containing plasmid pBWF1.8, four out of 80 wheat plants showed the typical symptoms of severe stunting characteristic of the wheat dwarf disease. Samples of infected leaves were probed for replicative forms of WDV as described. Figure 5 shows the result of a Southern experiment; all forms of replicative WDV DNA were detected.

Leafhopper transmission. One diseased plant was used as a feeding source in a leafhopper-transmission experiment using nonviruliferous *P. alienus*. After a 2-day acquisition period, four insects were transferred to each of 12 healthy test plants. All 12 test plants became diseased after 3 weeks.

WDV from France was similar to the WDV isolates from Sweden (WDV-S) and Czechoslovakia (WDV-C). Table 1 lists the sequence differences between WDV-F and the northern and eastern European isolates WDV-S and WDV-C. A total of 58

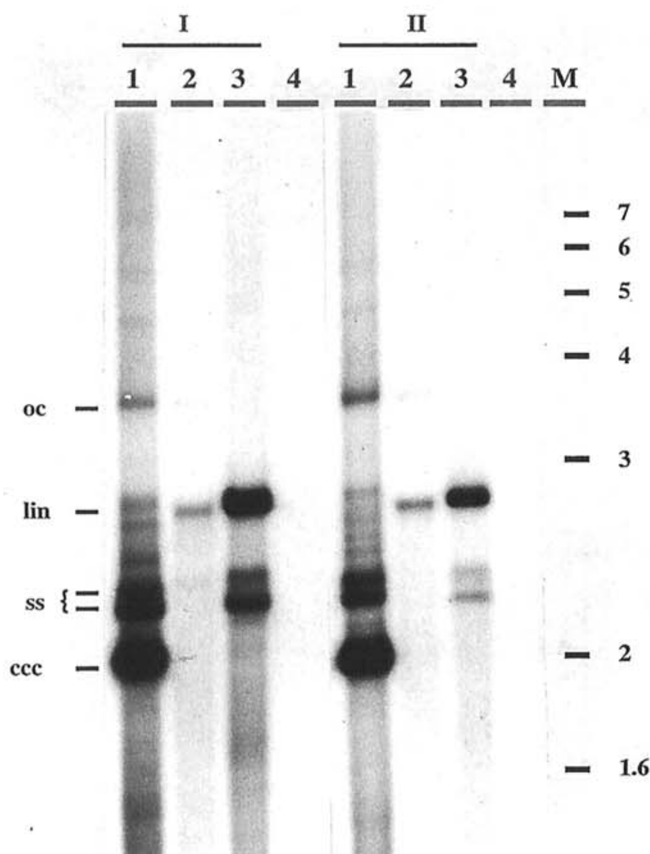

Fig. 5. Replication of wheat dwarf geminivirus from France (WDV-F) in *Triticum aestivum* cv. Briscard plants. Five micrograms of total DNA isolated from two different agroinoculated wheat plants (I and II) were separated and analyzed as described for Figure 4. Lane 1, native viral DNA forms in an undigested DNA sample; lane 2: DNA digested with S1-nuclease; lane 3: DNA digested with *Sst*I endonuclease; lane 4: DNA from a healthy wheat plant. The positions of the open circular (oc-), covalently closed circular (ccc-), and linear (lin) double-stranded forms of the respective DNAs are indicated. The positions of the single-stranded (ss-) circular and linear DNAs that are resolved in this gel are indicated. Lane M: position and sizes (kilobases) of the fragments of the 1 kb DNA-ladder (Gibco BRL).

positions with sequence differences among the three isolates were identified. Of these, 21 were unique in WDV-F and identical in WDV-S and WDV-C. At 18 positions the sequence of WDV-F was the same as in WDV-S and at 19 positions the same as in WDV-C, 14 of which cluster in the right half of the WDV genome. At only one position (base 118 in the large intergenic region [LIR]) did all three isolates differed. Note that the nomenclature of the ORFs was according to the recommendations of the 1st International Symposium on Geminiviruses, Almeria, Spain, 1994.

DISCUSSION

Until recently, the occurrence of WDV had been only described for northern and eastern Europe. The most recent report of an identification of WDV was from Hungary (4). In 1989 to 1990, crop losses due to the disease 'pieds chetifs' caused by a hitherto unidentified agent, but provoking symptoms resembling wheat dwarf, were reported from the central regions of France (9,24). We unequivocally identified the causative agent of the disease as an isolate of wheat dwarf geminivirus, WDV-F.

To clone the WDV genome, we developed the GA-method, a rapid isolation method for ccc-DNA molecules from plant tissue. The method relied on the denaturing power of guanidium salts (hydrochloride or thiocyanate) (10) and the trapping of denatured high-molecular-weight DNA in a potassium dodecylsulfate precipitate (3). The GA-method proved very efficient for the purification of geminivirus DNA free of high-molecular-weight chromosomal DNA. This was of considerable advantage for the rapid cloning of infectious viral genomes from a large number of samples. The GA-method provided an equally rapid alternative to polymerase chain reaction amplification prior to cloning (6,38) and avoided the introduction of undesired and potentially lethal sequence alterations into the virus genome, as discussed by Patel et al. (38). Using the GA-method, the infectious genomes of several geminiviruses from both grasses and herbaceous plants were cloned: WDV-ER, a barley-specific isolate of WDV (S. Schaefer and B. Gronenborn, *unpublished data*); a new isolate of TYLCV (M. Bendahmane and B. Gronenborn, *unpublished data*); and the genome of WDV-F (this study).

The infectivity of the cloned WDV-F DNA was confirmed by agroinoculation. To speed up and simplify the inoculation procedure, we used vacuum infiltration. Originally, this technique had been described for mass transformation of *Arabidopsis thaliana*, but we successfully applied it to agroinoculate wheat plants with cloned virus DNA. Although the rate of infection was rather low (5%) in the initial experiment, the simplicity and speed of the procedure that obviated the need to inject numerous small seedlings near the meristem easily compensated for this drawback. Also, modulating vacuum strength and using *Agrobacterium* strains more efficient on wheat (31) may have improved the infection rate.

The sequence of the DNA of WDV-F was 98.7% identical to WDV-S and 98.6% identical to WDV-C. Curiously, in all three WDV isolates sequenced an ORF of complementary strand polarity (C5) overlapping V2 and V1 was present. No data on its significance are available to date. It should be noted, however, that no comparable ORF was found in WDV-ER, the barley-specific isolate of WDV from Czechoslovakia (23).

The sequence differences among WDV isolates were not scattered randomly over the viral genomes (Table 1). A cluster of similarity between WDV-F and WDV-C occurred at the amino-terminal region of the viral capsid protein. This region had been found to be the most variable in the capsid protein gene of many geminiviruses and by itself already represented a sufficient basis for phylogenetic comparisons within the Geminiviridae (37). At position 167, a C to G transversion created an additional AUG codon; translation initiation here would have added two more amino acids to the V2 protein. Also, an additional T in the small

intergenic region (SIR) was common between the Czech and the French isolates of WDV. If only the capsid gene was considered, WDV-F was closer to WDV-C than to WDV-S, whereas, on the basis of their overall sequence variation, the three isolates were about equidistant.

Two independent clones of WDV-S have been sequenced (17,30), and these two differ at positions 2,448 and 2,697. These positions, however, were identical in WDV-F and WDV-C, as well as in the WDV-S sequence cited by Gronenborn and Matzeit (17). The difference at position 2,448 would have altered the corre-

TABLE 1. Sequence differences among wheat dwarf geminivirus isolates from France (WDV-F), Sweden (WDV-S), and Czechoslovakia (WDV-C)

Position (WDV-F)[a]	Gene (ORF)[b]	WDV-F base, amino acid[c]	WDV-S base, amino acid[c]	WDV-C base, amino acid[c]
46	LIR	C	Δ	Δ
92	LIR	*T*	G	*T*
113	LIR	G	T	T
118[d]	LIR	T	G	C
125	LIR	G	C	C
145[e]	LIR	Δ	A	A
159	LIR	G	T	T
167[f]	LIR/V2?	G Met	C	C
185	V2	C	A	A
232	V2	A Asn	T Ile	T Ile
281	V2	*C*	T	*C*
450	V1	*A*	G	*A*
453	V1	*T*	G	*T*
457	V1	*C*	A	*C*
459	V1	*C* *Ile*	G Met	*C* *Ile*
465	V1	*G*	A	*G*
468	V1	*A*	T	*A*
517	V1	**G** **Ala**	**G** **Ala**	A Thr
936	V1	T	C	C
957	V1	*A*	G	*A*
990	V1	*G*	T	*G*
996	V1	**A**	**A**	C
998	V1	G	A	A
999	V1	*C* Ser	T Asn	*C* Asn
1,089	V1	*C*	T	*C*
1,113	V1	**T**	**T**	C
1,122	V1	*C*	T	*C*
1,173	V1	**T**	**T**	C
1,268	SIR	*T*	Δ	*T*
1,288	SIR	**T**	**T**	C
1,303	SIR	**G**	**G**	C
1,388	C1-2	*A*	G	*A*
1,421	C1-2	**C**	**C**	T
1,423	C1-2	**T** **Thr**	**T** **Thr**	A Ser
1,453	C1-2	*C* *Ala*	T Thr	*C* *Ala*
1,493	C1-2	**C**	**C**	T
1,550	C1-2	T	G	G
1,553	C1-2	*T*	C	*T*
1,709	C1-2	A	G	G
1,849	C1-1	T	G	G
1,918	C1-1	T	C	C
1,933	C1-1	A	T	T
2,035	C1-1	A	G	G
2,107	C1-1	G	A	A
2,215	C1-1	**G**	**G**	A
2,278	C1-1	**T**	**T**	A
2,280	C1-1	**A**	**A**	G
2,281	C1-1	**G**	**G**	A
2,448[g]	C1-1	*G*	C/G* Gln/Glu*	*G*
2,584	LIR	G	C	C
2,611	LIR	G	C	C
2,612	LIR	**A**	**A**	T
2,670	LIR	**A**	**A**	T
2,683	LIR	**T**	**T**	G
2,693	LIR	**A**	**A**	T
2,697[g]	LIR	*G*	T/G*	*G*
2,711	LIR	T	C	C
2,718	LIR	**C**	**C**	T

[a] Base numbering refers to the coordinates of the WDV-F sequence. Base 1 is the first nucleotide of viral strand DNA synthesis, beginning in the conserved nonamer TAATATT[1]AC.

[b] Open reading frames (ORF) are as in Figure 3, LIR = large intergenic region; SIR = small intergenic region.

[c] Bases unique in WDV-F but common between WDV-S and WDV-C are given in stenciled letters (e.g., A,C,G,T), bases common between WDV-F and WDV-C are given in italic letters (e.g., *A,C,G,T*), bases common between WDV-F and WDV-S are given in bold letters (e.g., **A,C,G,T**). The resulting amino acid changes are indicated the same way. In case these result from a double base change, they are listed at base number 3 of the respective codon.

[d] Base 118 is different in all three WDVs.

[e] 145 denotes the position of a single base deletion in WDV-F; the position is not counted.

[f] Base 167 creates an additional methionine codon two codons ahead of the initiation codon of V2.

[g] Bases that differ between the sequence of WDV-S as deposited under X02869 (*) and the WDV-S sequence cited in (17).

sponding amino acid of the C1-1 gene product from glutamine to glutamic acid at a position in which in the C1 and AL1 proteins of all geminiviruses either positively charged amino acids (H, K, R) or those with an amide group in the side chain (N, Q) were strictly conserved. We, therefore, considered the occurrence of a glutamic acid to be unlikely, and evidence for the infectivity of that clone (30) needed to be presented. Only a single nucleotide position (position 118 in intergenic region) was fully variable among the three isolates of WDV.

When one compares the variation among different isolates of MSV, the overall sequence diversity among MSV isolates from South Africa (MSV-S), Nigeria (MSV-N), and Kenya (MSV-K) is 2.2 to 2.5% (25), slightly higher than the variation among the WDV isolates. However, 18 bases are completely variable among the MSV isolates as opposed to only one fully variable base in the three WDV isolates. A recent and more extended comparison of MSV sequences confirmed these results (5). The maximal variability in sequence over a stretch of 990 nucleotides is 3.2%. Another interesting example illustrating the evolution of a geographically isolated geminivirus is represented by sugarcane streak virus from Mauritius as compared to the one from Nigeria (40); in a coding sequence of only about 250 bases, 25 (10%) silent base changes had occurred. Similarly, the MSV isolates from Mauritius and Rèunion could be grouped in a distinct subcluster when compared with the MSV isolates from the rest of Africa based on capsid gene sequences (5).

The high degree of similarity among the WDV isolates may have suggested that they were introduced into different regions in Europe, rather than evolved within a given habitat for a longer period of time. A recent example of introduction of a geminivirus into a new environment has been demonstrated for TYLCV in the Dominican Republic, in which the local virus appears to be an isolate of the Near East TYLCV (33). Alternatively, the sequence drift in European wheat geminiviruses may, for unknown reasons, have been less pronounced than the one of geminiviruses endemic in African grasses and invading the monocot crops of the region. In the case of TYLCV isolates, for instance, a significantly higher sequence variation than that among the WDV isolates was observed: the three TYLCV isolates from the West Mediterranean cluster (36) differed between 7.2 and 11.4%. The same degree of diversity (8%) becomes apparent when the sequence of an ancient TYLCV isolate from the Near East, conserved as frozen sample since the sixties, was sequenced and compared to the more recently cloned TYLCV-I from Israel (1,33).

A somewhat intermediate position in sequence divergence is represented by the California strain and the hypervirulent CFH strain of beet curly top virus (BCTV) whose sequences differ by 17.5% (43). The same holds true for the WDV isolates and the barley isolate of wheat dwarf geminivirus (WDV-ER) (27); they exhibit a 16.4% overall sequence diversity (23). WDV-S and WDV-ER do complement each other for replication; i.e., the viral Rep protein of WDV interacts with the heterologous intergenic region of WDV-ER and vice versa (F. Heyraud-Nitschke and B. Gronenborn, *unpublished data*). BCTV strain Logan (identical to the California strain) and strain Worland do not cross complement (8). Also, the more distant TYLCV-S from Sardinia and TYLCV-I (25% divergence) do not complement the replication protein mutants of each other (21).

Based on the geminivirus sequences available in 1994, information about their evolution on a global scale and propositions for their taxonomy have been developed (37,39). The local or regional evolution of geminivirus populations as reflected by their sequence diversity is probably best represented by the studies on bean-infecting geminiviruses from the Caribbean and Central America (see 15 and references therein). These data, however, do not provide an explanation of the remarkable sequence conservation of the European WDVs. In this context it will be interesting to determine the sequence of a recently identified barley-specific isolate of WDV in France (H. Lapierre and B. Gronenborn, *unpublished data*) and to compare it to the barley strain (WDV-ER) from Czechoslovakia. This may shed further light on the issue of WDV diversity and spread within Europe.

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