Replication of wheat dwarf virus DNA in protoplasts and analysis of coat protein mutants in protoplasts and plants

Crispin J.Woolston, Helen V.Reynolds, Nicola J.Stacey and Philip M.Mullineaux

John Innes Institute and AFRC Institute of Plant Science Research, Colney Lane, Norwich NR4 7UH, UK

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ABSTRACT
The replication of wheat dwarf virus (WDV) in protoplasts derived from a *Triticum monococcum* suspension cell system was investigated. The production of circular viral double-stranded DNA (dsDNA) forms consistent with the replication of the viral genome was observed. In comparison to whole plants, the production of viral single-stranded DNA (ssDNA) was reduced, possibly due to only low levels of viral coat protein being produced in the protoplasts. Mutations introduced into the viral coat protein open reading frame (ORF) did not affect the ability of the viral DNA to replicate, and a deletion of ca. 400 bp was tolerated. However, these mutations abolished the infectivity of the viral genome when agroinoculated onto wheat plants, providing evidence that, contrary to the case for the bipartite geminiviruses, the coat protein is essential for infection by WDV.

INTRODUCTION
Wheat dwarf virus (WDV; 1) is representative of the monopartite geminiviruses which infect monocotyledonous plants (2,3), a group which includes maize streak virus (MSV; 4,5) and digitaria streak virus (DSV; 6). It has a single-stranded circular DNA genome of 2.7 kb (3,7) which is packaged within twinned quasi-isometric particles. Infected plants also contain double-stranded forms of the viral DNA (dsDNA) which may represent replicative intermediates (8,9). Infection of plants with cloned DNA of the monopartite geminiviruses is only possible using the process of agroinfection (10). This method has been used to determine the infectivity of MSV (11,12,13), WDV (7,14) and DSV (15).

The use of DNA viruses to investigate the molecular biology of virus–host interactions, and in cell transformation is exemplified by work with SV40 (16). Such studies have been greatly facilitated by the availability of techniques for the isolation, culturing and infection of single cells. Similar investigations of plant–virus interactions using the monopartite geminiviruses are limited by the absence of well characterised cell systems, but studies of the bipartite geminiviruses have been made using *Nicotiana* protoplasts (8,17). We have investigated the use of protoplasts derived from a *Triticum monococcum* suspension culture (18,19), which supports the replication of WDV (20), and have examined the replication of mutant genomes of WDV in this cell system and in plants. We find that the size of the viral genome can be reduced when in the cell system. We also find that the viral coat protein appears to be essential for the development of systemic symptoms in inoculated plants. This is contrary to the evidence presented for the bipartite geminiviruses African cassava mosaic virus (ACMV; 21,17) and tomato golden mosaic virus (TGMV; 22,23). However, evidence for the involvement of the viral coat protein gene in viral spread has recently been presented for MSV (24,25), and for another monopartite geminivirus, beet curly top virus (BCTV; 26).

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Table 1. Summary of the constructs used.

Plasmid Name	Description
pWDVK10D	dimer of wild-type WDV-CJI genome in pIC20R
pWDVΔ1D	dimer of *Mlu* 1 frameshift mutant in pIC20R
pWDVΔ2D	dimer of *Nsi* 1 to *Mlu* 1 deletion in pIC20R
pWDVΔ3D	dimer of *Nsi* 1 frameshift mutant in pIC20R
pJIT33	dimer of wild-type WDV-CJI genome in pBin19
pBinWDVΔ1D	dimer of *Mlu* 1 frameshift mutant in pBin19
pBinWDVΔ2D	dimer of *Nsi* 1 to *Mlu* 1 deletion in pBin19
pBinWDVΔ3D	dimer of *Nsi* 1 frameshift mutant in pBin19
pWDVMK1	monomer WDV-CJI in M13mp18, (−)sense specific
pWDVMK2	monomer WDV-CJI in M13mp18, (+)sense specific

MATERIALS AND METHODS

Construction of WDV coat protein mutants.

pWDVK10 is a clone of an infectious, monomeric WDV-CJI genome inserted into M13mp18 (7). Plasmid pJIT34 was derived by moving the WDV *Kpn* I unit genome from pWDVK10 into the *Kpn* I site of pIC20R (27). Plasmid pWDVK10D was derived at the same time by cloning a tandem dimer of the WDV *Kpn* I fragment into the same site of pIC20R. For the construction of the frame-shift mutants pJIT34 was digested with either *Mlu* I or *Nsi* I and the single-stranded overhangs either filled in (*Mlu* I) or digested away (*Nsi* I) with T4 DNA polymerase I (Boehringer) using standard conditions (28). For the construction of the deletion mutant, pJIT34 was digested with both enzymes and treated with T4 DNA polymerase I. Blunt-end ligation was followed by transformation into *E. coli* JM83 ^{recA} (7) and transformants were screened for the loss of either the restriction sites or the intervening fragment. Nucleotide sequencing confirmed the nature of the mutations. Each mutant was excised as a *Kpn* I fragment, purified and cloned into *Kpn* I cut pIC20R under conditions favoring the formation of dimeric inserts. The mutants were also cloned as tandem dimers into the *Kpn* I site of pBin19 (29). The plasmids obtained are detailed in Table 1.

For the strand-specific probing of protoplast DNA samples, we constructed pWDVMK1, which consists of the *Kpn* I WDV monomer inserted into the *Kpn* I site of M13 mp18 such that the ssDNA is complementary to the (−) sense DNA strand of WDV. Clone pWDVMK2 has this insert in the opposite orientation such that it is complementary to the WDV (+) sense DNA strand

Maintenance of suspension cultures.

The *Triticum monococcum* cell culture was originally obtained from Dr. H. Lrz, Max-Planck-Institute, Cologne. Cultures were shaken at 60 rev min^{-1} at 25°C and maintained in 100 ml Ehrlenmeyer flasks in 75 ml of P10 medium (30). The flasks were subcultured weekly by the replacement of 25 ml with fresh medium.

Isolation of protoplasts.

5 ml of cells, 4−7 days after subculturing, were transferred to a 9cm petri dish and the medium removed. 15 ml of cleared, sterile enzyme solution (0.05% Driselase, 0.05% Cellulase (Onozuka) 0.05% Rhozyme, 0.025% Pectolyase Y23, 3.0 mM MES, 0.1% BSA (fraction V) pH 5.6) was added, the dish sealed and incubated in the dark for 16 hrs at 25°C. Filtration through successive gauzes of 250μm, 103μm and 50μm (Endecotts Ltd, London), was followed by addition of 20 ml of solution W (0.7 M mannitol, 10mM

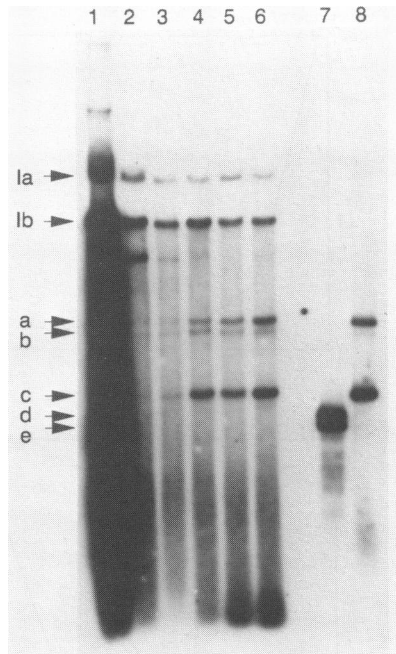

Figure 1. Autoradiograph of Southern blot, using a full-length WDV-specific probe, of DNA samples extracted from inoculated protoplasts at 0 days (1), 1 day (2), 2 days (3), 3 days (4), 4 days (5) and 5 days (6) post inoculation (dpi) with pWDVK10D. Lane 7 is viral ssDNA and lane 8 is dsDNA extracted from infected plants. Bands indicated are inoculum DNA (Ia and Ib), progeny dsDNA; o.c. (a), linear (b), s.c. (c), viral ssDNA (d,e; lane 7 only).

$CaCl_2$. Following centrifugation at 600 rev min^{-1} for 5 min (MSE Centaur) the supernatant was discarded and the pelleted protoplasts resuspended in 25 ml of solution W. After repeating this step the protoplasts were counted, divided into batches of 4×10^6 and pelleted as before.

Inoculation of protoplasts.

The inoculum DNA was added to the pelleted protoplasts (typically 50–100 μg in 100μl of 50% solution W). Following gentle mixing, 0.5 ml of PEG solution (30% PEG 6000, 3 mM $CaCl_2$, 0.7 M mannitol) was added and the protoplasts mixed gently for 15 seconds before addition of 5 ml of solution W. After incubation at room temperature for 20–30 minutes the volume was increased to 30 ml with solution W, and the protoplasts pelleted as before. Two further washes in 20 ml of solution W were followed by a final resuspension in 16 ml of medium C8IV (31). Protoplasts were cultured at a density of $1 \times 10^6 ml^{-1}$ in the dark at 28°C in parafilm sealed 5 cm petri dishes lined with a 2ml base of C8IV in 0.7% agarose.

Extraction of protoplast DNA.

The inoculated protoplasts were harvested, frozen at −20°C, thawed and resuspended in a 1.5 ml microfuge tube in 200 μl of lysis mix (1% SDS, 2 mM EDTA, 0.5 mg/ml Pronase (pre-digested)). The sample was ground in acid-washed fine sand and incubated at room temperature for 20–30 min. Extraction of the nucleic acids was performed by

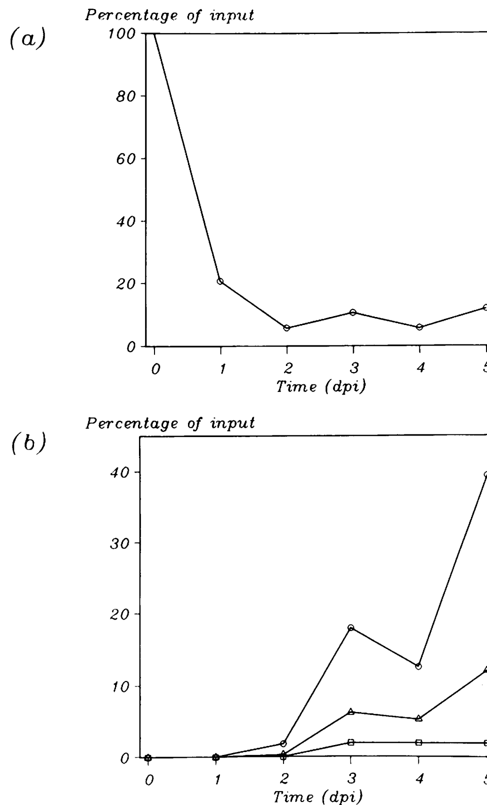

Figure 2. Graphical representation of a densitometric analysis of the autoradiograph shown in Fig. 1. Panel (a) shows the changes in amount of inoculum DNA over the course of the experiment. Panel (b) shows the changes in amounts of the progeny DNA forms (o.c. △, linear □, and s.c. ○). Results are expressed as the percentage of the input DNA amount at t=0.

adding an equal volume of modified Kirby phenol (32) followed by addition of an equal volume of 1:1 phenol:chloroform. The aqueous phase was transferred to a fresh tube, centrifuged to pellet insoluble material and then extracted twice more with phenol:chloroform. The nucleic acids were then precipitated by the addition of 0.1 vol 3 M Sodium acetate pH. 5.2, 2.5 vol ethanol and pelleted by centrifugation. The washed pellet was resuspended in 100 μl of TE (10 mM Tris-Cl pH. 8.0, 1 mM EDTA).

Analysis of samples.

The protoplast samples were analysed by electrophoresis of 5 – 10 μl of the purified nucleic acids (equivalent to $5\times10^4-10^5$ protoplasts) on 1% agarose gels in TAE buffer (28), at 100 mA for 4 hrs. Southern transfers were performed as previously detailed (28). Filters were probed with oligo-labelled (33) WDV-CJI DNA using standard conditions (28). Strand-specific hybridisation was carried out using the sandwich hybridisation method (34).

The intensities of the bands on the exposed autoradiographs were determined by scanning densitometry using a Joyce-Loebel Chromoscan 3.

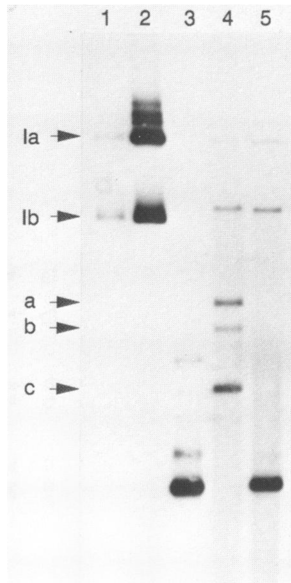

Figure 3. Autoradiograph of Southern blot, using a full-length WDV-specific probe of inoculum DNA (1–3) or inoculated protoplast DNA (4–5) either undigested (1,4), digested with *Mbo* I (2,5), or *Sau* 3A (3). Bands marked are as in legend to Fig. 1.

Plant inoculations.

The agroinoculation method (10) for introduction of geminivirus DNA into plants and the subsequent dot-blotting of plant tissue were performed as previously detailed (7). Each construct tested was inoculated onto 100 plants. *Agrobacteria* used for inoculations were assayed for the presence of the correct plasmids by Southern blotting.

RESULTS.

We have previously reported the infectivity of cloned WDV-CJI by agroinfection (7). This infectious, full-length WDV-CJI genome was inserted as a tandem dimer into pIC20R to form pWDVK10D. Inoculations of this construct into protoplasts of *Triticum monococcum* lead to the production of novel, WDV-specific DNA forms. These show the same electrophoretic mobilities as the double-stranded forms of the viral DNA found in WDV infected wheat plants, and were first detectable 24 hours after inoculation (Fig 1), increasing in abundance during the course of the experiment (Fig 1 and 2). Restriction enzyme digestion of these WDV-specific forms confirmed that they were the dsDNA forms (open-circular, linear and supercoiled respectively) of the circular viral genome (data not shown). Inoculated protoplasts started to divide at about 3 days after inoculation. By 5 to 6 days after inoculation this had reached a maximum such that 10% of the protoplasts were undergoing division. After 8 days in culture protoplast viability showed a significant decline.

Degradation of the inoculum DNA appears to occur immediately following inoculation, giving rise to the heterogeneous smear on gel electrophoresis of WDV-specific DNA in

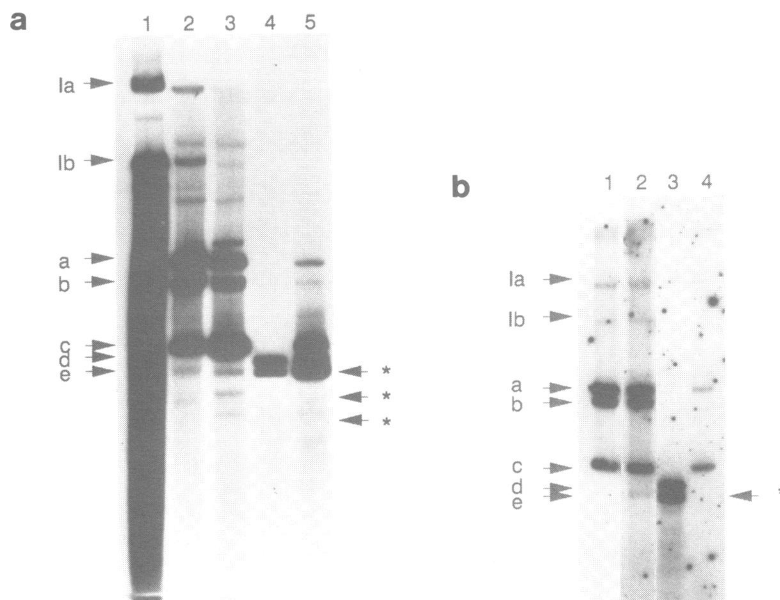

Figure 4. (a) Autoradiograph of Southern blot, using a full-length WDV-specific probe, of DNA samples extracted from pWDVK10D inoculated protoplasts at 0 (1), 3 (2), and 6 (3) days post-inoculation. Lane 4 is viral ssDNA and lane 5 total DNA from WDV infected plants. Bands indicated on the left are as previously. The upper band of those marked on the right (*), visible in lane 3, co-migrates with one of the ssDNA bands. (b) Autoradiograph of Southern blot, probed with a WDV (−) sense-specific (1), or WDV (+) sense-specific (2,3,4) probe, of the 3 dpi sample used in lane 3 of Fig. 5a. Lane 3 is viral ssDNA and lane 4 is dsDNA from WDV infected plants. The bands marked with arrows on the left are as previously described. The band on the right (*) is visible in lane 2 but not in lane 1.

the 0 day timepoint. However, approximately 5% of the input DNA is maintained apparently intact in subsequent timepoints (Figs. 1 and 2). This probably represents the amount of DNA which is protected by delivery into the protoplasts and from which the novel WDV-specific forms are derived. Similar observations have been made for the replication of ACMV DNA in protoplasts of *Nicotiana plumbaginifolia* (8).

The methylation pattern of these viral-specific novel DNA forms was investigated to determine that they had been synthesised *de novo* in the protoplasts and had not been produced due to an accumulation of input DNA which had undergone homologous recombination to yield non-replicating WDV monomeric circles. The input and progeny DNA was digested with either *Sau* 3A or *Mbo* I, both of which cut at the motif 'GATC', but which are sensitive to methylation at different positions on this recognition sequence which occurs four times in the WDV-CJI genome. Input DNA was resistant to *Mbo* I digestion but was fully cut by *Sau* 3A (Fig 3), indicating that a high percentage of the sites were blocked by methylation of the adenine residue, but that the cytosine residue was not methylated. However, the progeny DNA forms could be cut with *Mbo* I (Fig. 3). This absence of methylation at the adenine demonstrates that the progeny DNA must have been synthesised *de novo* in the protoplasts, since demethylation in the absence of DNA replication is not known to occur.

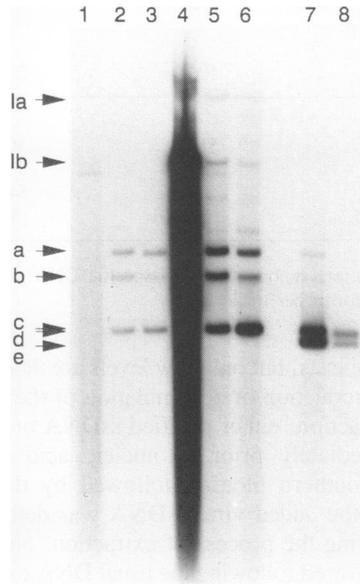

Figure 5. Autoradiograph of Southern blot, using a full-length WDV-specific probe, of DNA samples extracted at 0 (1 and 4), 3 (2 and 5) and 6 (3 and 6) dpi from protoplasts inoculated with either 20μg (1−3) or 100μg (4−6) of pWDVK10D DNA. Lane 7 is total DNA from WDV infected plants and lane 8 is viral ssDNA.

The resistance of the residual input DNA forms in the protoplast samples to digestion with *Mbo* I also shows that their maintenance is not caused by low level replication due to the presence on the plasmid of the WDV origin of replication (Fig 3). The possibilty that the single WDV origin remaining on the input DNA after excision of the WDV monomer could lead to DNA replication is discounted by the fact that we do not detect this DNA form. This may be due either to the intervening bacterial sequences exceeding some size limitation of the WDV replicon or to the presence of the bacterial origin interfering with replication in the plant.

Novel DSV-specific DNA forms could not be detected during analysis of protoplasts which had been inoculated with a tandem dimer of the DSV genome (15 ,data not shown). This indicates that this virus, whilst having an overlapping host range with WDV, was not capable of replication in the *Triticum monococcum* protoplasts. We take this as evidence that the WDV DNA forms arise due to replication of the WDV genome, since if these forms arose due to homologous recombination, one would also expect them to occur in protoplasts inoculated with the tandem DSV dimer.

The viral single-stranded DNA, which is present in infected tissue at an abundance at least equal to the amount of dsDNA (Fig. 7) did not appear to accumulate in the WDV inoculated *Triticum monococcum* protoplasts, but extended exposure of autoradiographs revealed several minor viral-specific bands, one of which appeared to co-migrate with the viral ssDNA marker (Fig. 4a). To confirm the identity of this band the samples were hybridised with WDV strand-specific probes. The band was detected by probe pWDVMK2, which is specific for WDV (+)sense DNA, but not detected by probe pWDVMK1, which is specific for (−)sense WDV DNA (Fig. 4b). This indicates that production of WDV

Table 2. DNA forms in inoculated protoplasts

Timepoint (dpi)[a]	3		6	
Inoculum (μg DNA)	20	100	20	100
DNA form [b]				
open circular	35.8	31.2	36.7	20.9
linear	21.4	28.4	9.0	12.3
supercoiled	42.8	40.4	54.3	66.8
Total [c]	[3584]	[20440]	[3988]	[22456]

a. days post-inoculation
b. expressed as a percentage of the total amount of WDV-specific DNA present per track (Fig. 5).
c. total absorbance reading for all forms per track.

ssDNA can occur in the protoplasts, but only low levels are detected. In order to determine if this represented reduced production or accumulation of the ssDNA, or loss of ssDNA or virus particles during extraction, either purified ssDNA or virus particles were added to control protoplasts immediately prior to nucleic acid extraction. Samples were subsequently analysed by Southern blotting followed by densitometry analysis. This demonstrated that, although the added viral ssDNA was detectable in the final extract, about 90% had been lost during the process of extraction. No loss of ssDNA occurred, compared to the amounts recovered following the usual DNA extraction method (35), when purified virus particles were added to the protoplast sample.

The relationship between the amounts of input and progeny DNA was investigated by inoculations of equal numbers of protoplasts with either 100 μg or 20 μg of pWDVK10D DNA. Southern blot assay of the protoplast DNA extracted at 3 day intervals (Fig. 5), followed by scanning densitometry of the resultant autoradiograph demonstrated the total amount of WDV-specific progeny DNA to be directly proportional to the amount of inoculum (Table 2). This indicates that our conditions of inoculation are not saturating the protoplasts with viral DNA. These data also illustrated the change in relative amounts of the different viral DNA forms with time. Between the 3 and 6 day timepoints the linear and open circular forms of the DNA decrease and there is an increase in the amount of supercoiled DNA (Fig. 5, Table 2). This observation is supported by previous experiments (Fig. 1) which show a similar pattern, in that the supercoiled form of the DNA accumulates to the highest level and at the highest rate, with the other two forms staying either relatively constant or increasing at a lower rate (Fig. 2). It remains to be confirmed that this is a characteristic of the viral DNA replication mechanism and is not caused by different extraction efficiencies due to the differing ages of the protoplasts.

Three different mutations were introduced into ORF V2 (coat protein) of the WDV-CJI genome (Fig. 6) and assessed for their ability to replicate. None of the mutations altered any of the adjacent ORFs (V1 or C3). The modification of the *Mlu* I site (pWDV 1), which lies at position 1246 on the viral genome, within coat protein ORF, serves to shift the reading frame by -1 nucleotide, resulting in a putative extended polypeptide in which the C-terminal 9 amino-acids have been replaced by a different set of 13. Analysis of this alteration using the PEPTIDESTRUCTURE program of the University of Wisconsin genetics computer group (36) indicates that the normally hydrophobic terminus of the polypeptide is substantially altered in that it becomes weakly hydrophilic. Clone pWDV 3 was formed by alteration of the *Nsi* I site at position 812. This causes a reading frame shift of $+1$ in the coat protein cistron, leading to the production of a putative product

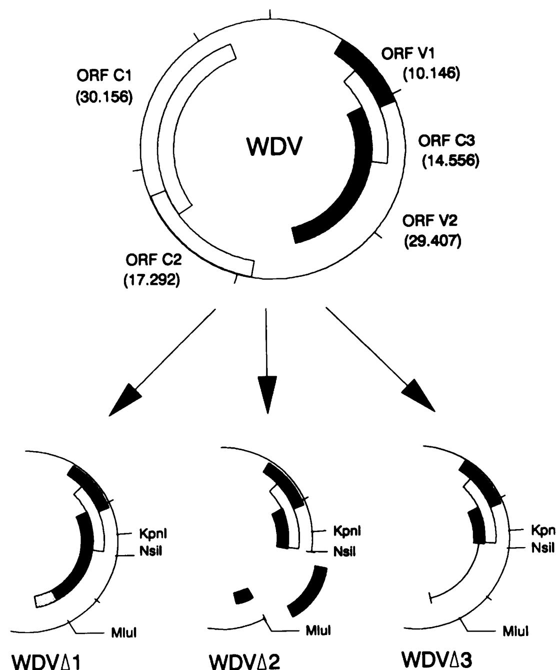

Figure 6. Diagram of the genome organisation of WDV (3,7) showing the virion sense (filled arcs) and complementary sense (unfilled arcs) ORFs (ORF C2 does not have an AUG codon at the 5′ end). The predicted sizes of the ORF products are shown in kilodaltons. The right hand sides of mutant genomes WDVΔ1, WDVΔ2 and WDVΔ3 are shown, indicating the extent and effect of the mutations. The region filled in half-tone is the extension to the coat protein gene incurred by the frameshift in WDVΔ1.

of 117 amino-acids, 143 amino-acids shorter than the wild-type product. Clone pWDV 2 was formed by the deletion of the 434 bp fragment which lies between the *Nsi* I and *Mlu* I sites. This reduces the size of the putative ORF V2 product by 146 amino-acids.

These mutant viral genomes were inoculated into *Triticum monococcum* protoplasts and DNA samples taken at intervals. In all three cases the inoculations led to the production of the novel viral-specific DNAs (Fig. 7) indicative of viral DNA replication. The amount of DNA produced was comparable to that produced by inoculations with the wild-type viral DNA construct (pWDVK10D). Clones pWDV 1 and pWDV 3 produced viral dsDNA forms which comigrated with wild-type viral dsDNA. Clone pWDV 2 produced the same banding pattern but displaced down the gel by an amount consistent with the deletion of ca. 400 bp. Restriction digestion of the progeny DNA in each case identified it as dsDNA and also demonstrated that the progeny restriction sites were consistent with the inoculum DNA (data not shown). Viral ssDNA was not detected in these samples, possibly due to the lack of viral coat protein.

The three WDV-CJI mutants were transferred into the vector pBin19 (29) as tandem dimers and then conjugated into *Agrobacterium tumefaciens* strain C58 (7). This allowed us to agroinoculate the mutants into young wheat plants and assess their ability to initiate an infection. DNA dot blot analysis of 300 inoculated plants distal to the inoculation site

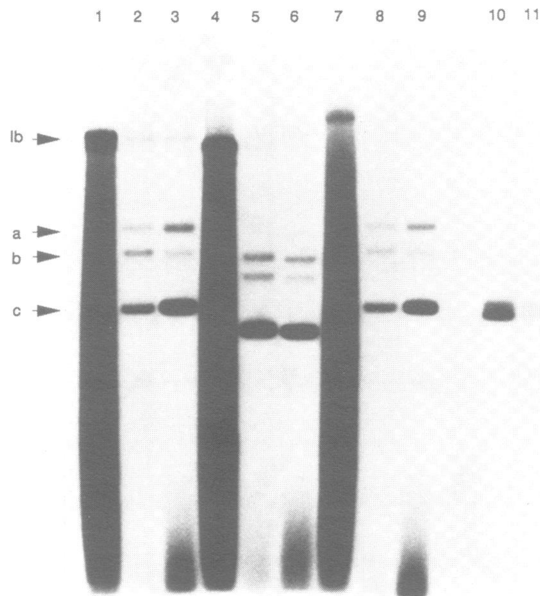

Figure 7. Autoradiograph of Southern blot, using a full-length WDV-specific probe, of DNA samples extracted at 0 (1,4,7), 3 (2,5,8) and 6 (3,6,9) dpi from protoplasts inoculated with either pWDVΔ1 (1–3), pWDVΔ2 (4–6) or pWDVΔ3 (7–9). Lane 10 is total DNA from WDV infected plants, and lane 11 is viral ssDNA.

consistently failed to reveal any viral DNA, demonstrating that the mutation of the coat protein ORF substantially affected the ability of the virus to initiate an infection following agroinoculation. 44% of the control plants inoculated with an intact WDV-CJI dimer in pBin19 (pJIT33) developed symptoms and showed positive for viral DNA by dot blotting. Sampling the plants inoculated with the coat protein mutants at later times to detect delayed infection was similarly unsuccessful. Sampling for viral DNA at the site of inoculation to determine DNA replication, as reported for MSV (24,25) was not undertaken as our results from the protoplast inoculations yielded the same information.

DISCUSSION

We have demonstrated that WDV genomes in which the coat protein ORF has been altered by either a reading frame shift or by deletion retain their ability to replicate in protoplasts, at the same level as wild-type viral DNA, but are not able to initiate infection of whole plants when delivered by agroinoculation. The deletion mutant shows no size reversion in protoplasts, an observation consistent with previous reports (17). The analysis of these mutant genomes has been facilitated by the characterisation of a *Triticum monococcum* protoplast system in which inoculation with cloned tandem dimers of WDV leads to the replication of the viral circular dsDNA. The viral replication implies escape of the genome from the bacterial vector prior to replication, since we detect no replication of the intact inoculum DNA, nor of any intermediate. This viral escape, possibly facilitated by homologous recombination, is likely to be enhanced by the presence of dimeric genomes

which provide a 2.7 kb direct repeat. The DNA forms produced as a result of the viral replication are consistent with those produced by the virus in infected plants, with the exception of the viral ssDNA.

The low levels of this ssDNA observed in the protoplasts may be due in part to losses during the extraction procedure of unencapsidated ssDNA. However, our observation that encapsidated ssDNA is efficiently extracted from the protoplasts, suggests that the encapsidation of the ssDNA should lead to its stabilisation and recovery. Possibly the ssDNA is not being sequestered into the viral particles. The expression of WDV ORF V2 can be detected in *Triticum monococcum* protoplasts when using the bacterial chloramphenicol acetyl transferase gene (*cat*) as a reporter, although we are unable to detect viral coat protein by ELISA, western blotting or immunofluorescence following inoculations with cloned wild-type virus (C.J.W. and H.R. unpublished data). It has previously been suggested, by analogy with ϕX174 (37), that the accumulation of ACMV ssDNA only occurs later in infection when the products necessary for its sequestration are being expressed. Prior to this the naked ssDNA acts as a template for (−) strand synthesis leading to the production of more dsDNA (8). This hypothesis is supported by the observation that coat protein mutants of ACMV show reduced levels of ssDNA when inoculated into protoplasts (17). It is likely that a similar situation occurs with WDV in *T. monococcum* protoplasts and that we do not detect much ssDNA as the viral ORFs involved in its sequestration are not highly expressed early in the timecourse of our experiments. It should be emphasised, however, that the amount of ssDNA found in infected plants may represent the accumulation of at least 20 days synthesis, whereas the protoplasts were only sampled to 6 days post-inoculation.

Our results from the inoculations of ORF V2 (coat protein gene: Fig. 6) mutants of WDV demonstrate that this ORF is inessential for dsDNA replication and also that the WDV genome will tolerate a deletion of ca. 400 bp, at least in protoplasts, with the size of the replicon remaining at 2.3 kb. This observation is consistent with the identification of a subgenomic form of the WDV genome in infected plants (38), deleted in the region of the coat protein gene. Previously it was not possible to determine if this sub-genomic form of the genome had lost sequences vital for DNA replication, and was maintained by the provision of these functions *in trans* from the intact viral genome. Our data would suggest that the subgenomic DNA form reported could replicate autonomously. Agroinoculation of whole plants with the viral mutants, however, demonstrates that the product of ORF V2 is essential in the normal development of infection of the virus, assuming that successful agroinoculation is, in this case, bound by the same criteria as is inoculation by the leafhopper vector. Comparison of the computer predictions of the protein secondary structure of the wild-type ORF V2 product and the potential product of ORF V2 from mutant pWDV 1 suggests that this alteration may have a profound effect on the hydrophilicity of the extreme C-terminal portion of the protein. This portion of the coat protein gene of several geminiviruses is conserved (39), and it is likely that its modification affects the function of the protein such that infectivity is lost.

This observation contrasts with similar experiments using ACMV (17) and TGMV (22,23), in which the coat protein ORF could be interrupted without loss of infectivity. In the case of ACMV directly infected into *N. benthamiana* it is essential that the natural genome size is maintained (17), whereas TGMV agroinoculated into *N. tabacum* will tolerate size modification. To what extent this difference is fundamental to the viruses or to their adaptation to the host plant remains to be determined (40), but studies have shown that

neither the different inoculation mechanism nor the host is responsible (41), and it has been suggested that ACMV may exhibit a size-limited spread (17).

The evidence from our experiments demonstrates that the coat protein gene of WDV plays some role in the infection process, possibly in the spread of the virus from cell-to-cell. Similar conclusions from mutagenesis experiments involving MSV have been made (24,25). ACMV and TGMV have bipartite genomes, and factors influencing cell-to-cell spread of the virus have been mapped to DNA 2 of ACMV (42). Given the absence of a DNA 2 component in the monopartite viruses, one might predict that determinants for their spread may lie within the coat protein ORF.

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