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Wheat dwarf virus, a geminivirus of graminaceous plants needs splicing for replication

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By analysing mRNAs with the polymerase chain reaction (PCR) and by studying *in vitro* generated mutants we have identified an intron in the genome of wheat dwarf virus (WDV), a geminivirus of cereals. Polypeptides whose expression is essential for the replication of the viral DNA have been defined. They are encoded by two distinct overlapping open reading frames (ORFs). The joining of these two ORFs by deletion of the intron as well as the introduction of a frameshift mutation within the intron do not prevent replication of the viral genome in suspension culture cells. In contrast to WDV, the geminiviruses of dicotyledonous plants possess a single continuous ORF, highly homologous to the two individual ones of WDV. We propose that mRNA splicing is a common feature of all geminiviruses of the Gramineae and might contribute to their host class specificity. The existence of a functional intron is a novel finding for the plant viruses.

Key words: cereals/intron/polymerase chain reaction/single-stranded DNA virus/viral vector

Introduction

Caulimoviruses and geminiviruses are the known two groups of DNA viruses of plants (Goodman, 1981; Shepherd and Lawson, 1981). The geminiviruses are characterized by twinned capsids which contain a single molecule of circular single-stranded DNA (Goodman, 1977; Harrison *et al.*, 1977). Their genomes are either monopartite or bipartite, consisting of one or two circles of ~2.6–3.0 kb (Stanley, 1985). Geminiviruses with a bipartite genome are transmitted by whiteflies, those with a monopartite one by leafhoppers (Harrison, 1985). The DNAs of several members of both viral subgroups have been sequenced: Cassava latent virus (CLV) (Stanley and Gay, 1983), tomato golden mosaic virus (TGMV) (Hamilton *et al.*, 1984), bean golden mosaic virus (BGMV) (Howarth *et al.*, 1985) of the whitefly transmitted subgroup, and beet curly top virus (BCTV) (Stanley *et al.*, 1986), different isolates of maize streak virus (MSV) (Howell, 1984; Mullineaux *et al.*, 1984; Lazarowitz, 1988), wheat dwarf virus (WDV) (MacDowell *et al.*, 1985), digitaria streak virus (DSV) (Donson *et al.*, 1987) and chloris striate mosaic virus (CSMV) (Andersen *et al.*, 1988) of the leafhopper transmitted subgroup. Transcripts have been mapped on both strands revealing that the genome is transcribed bidirectionally (Morris-Krsinich *et al.*, 1985; Townsend *et al.*, 1985).

Wheat dwarf virus (WDV) belongs to the single component geminiviruses and infects a variety of grasses including most cereals (Vacke, 1972; Lindsten *et al.*, 1980). We have cloned and sequenced the DNA of three different isolates of WDV, mapped the transcripts and analysed *in vitro* generated mutants of the different ORFs (Schalk *et al.*, in preparation; Gronenborn and Matzeit, 1989).

The comparison of potential proteins of wheat dwarf virus with those of the geminiviruses of dicotyledonous plants reveals that the deduced amino acid sequence of two individual ORFs of minus-strand polarity, the 30 kd ORF III and 17 kd ORF IV, are highly homologous to a single continuous ORF, with a coding potential for a 40 kd protein, on DNA A (DNA 1) of the dicot geminiviruses (Figure 1a and b). DNA A of the geminiviruses with a divided genome is capable of autonomous replication as a single circle (Rogers *et al.*, 1986; Townsend *et al.*, 1986). The regions of amino acid homology of WDV are interrupted by a sequence which has no counterpart within the 40 kd protein sequence of the dicot geminiviruses. In order to obtain the optimal sequence alignment, ORF IV of WDV has to be moved towards the amino terminus of ORF III (see Figure 1a and b).

The question arises of how ORF III and IV of WDV are expressed. Do they encode two individual polypeptides, or do they yield, by whatever mechanism, a single fused polypeptide analogous to the geminiviruses of dicot plants? Or is the expression of both individual polypeptides and a fusion product required?

Mechanisms by which a single polypeptide could be expressed from two distinct ORFs are frameshift suppression or splicing (Green, 1986; Craigen and Caskey, 1987).

Results

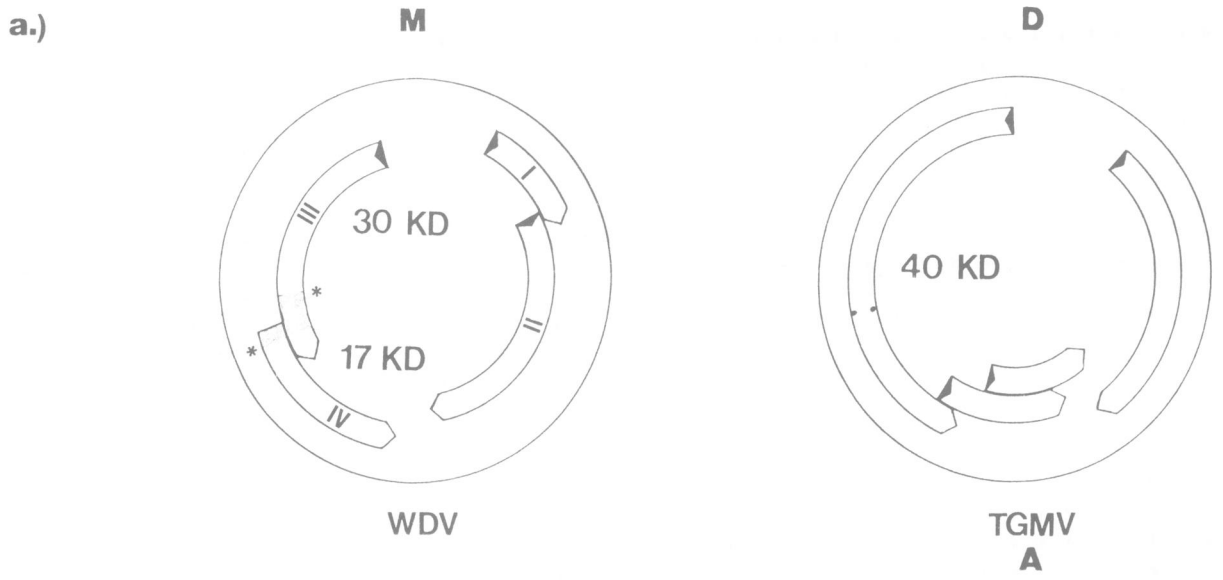
Introduction of a frameshift mutation

Inspection of the DNA sequence revealed that splice donor and acceptor sequences are located near the borders of the non-homologous sequence on the DNA of WDV (Figure 1a, shaded box). A splicing of the sequence indicated in Figure 1e would lead to an mRNA with a continuous reading frame for a polypeptide of 41 kd, and would explain how ORF IV of WDV which does not possess an ATG codon near its N-terminus, is translated.

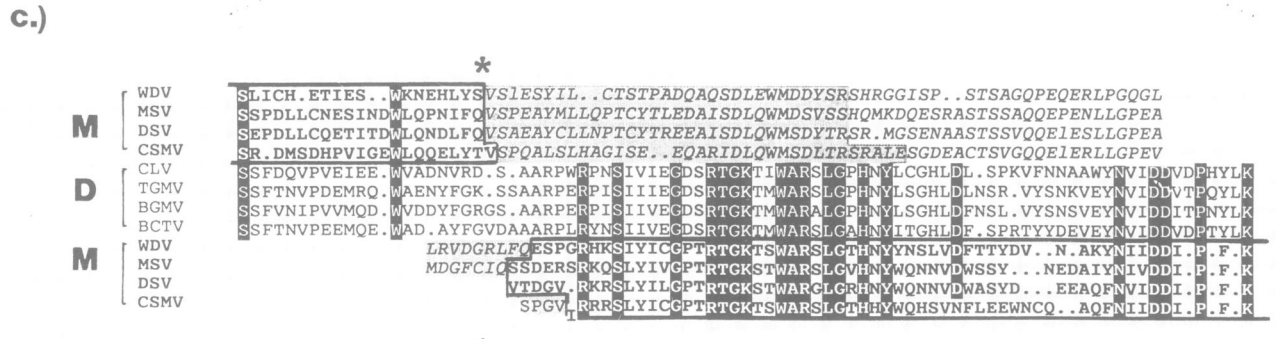
To test the 'intron hypothesis' we introduced two additional bases into the sequence of the presumed intron, 52 codons upstream of the ORF III stop codon. Duè to this mutation, the frame of ORF III is changed to a third frame and terminates prematurely at the beginning of ORF IV (Figure 1d; lane marked 'FSi'). The mutation was designed to create a unique *Xho*I site in order to be easily detected (Figure 2B).

Open reading frames required for replication of WDV

The influence of mutations on the replication of WDV DNA was tested by transfecting protoplasts of *Triticum mono-*


a.)
M
D
30 KD
17 KD
40 KD
I
II
III
IV
WDV
TGMV
A


b.)
HOMOLOGY:
M 30 KD
D 40 KD
M 17 KD


c.)
M
WDV
MSV
DSV
CSMV
D
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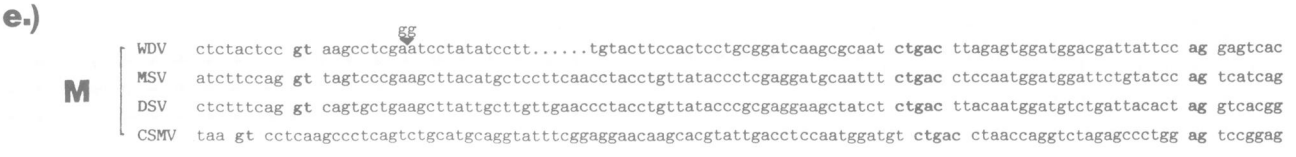
CSMV


INTRON
d.)
WDV
III
IV
FSi


e.)
M
WDV
MSV
DSV
CSMV

coccum suspension culture cells by the respective DNAs (Figure 2). Mutants of ORF I and ORF II do replicate (Matzeit *et al.*, in preparation; see also Figure 2c, lane 10). However, deletion mutants of ORF III and ORF IV (Figure 2A; stippled areas) do not replicate. For example (Figure 2a, lane 1), no replicative forms of viral DNA were detectable following transfection with DNA of the WDVΔIV mutant. Also mutants that affect protein expression, as for instance a mutant which disrupts only the ATG codon of ORF III (ATG^-), or a frameshift mutant of ORF IV (FS 75 IV), fail to replicate. Consequently, the expression of protein encoded by both ORFs is essential for the replication of WDV DNA in plant cells. Furthermore, mutants of the individual ORFs do not complement each other in co-transfection experiments, which indicates that the individual proteins alone are not sufficient to support replication (data not shown).

Replication of the frameshift mutant

In contrast to the various other mutants, which affect the region of ORFs III and IV, the frameshift mutant in the 'intronic region' of ORF III (FSi) replicates, as is demonstrated by the appearance of covalently closed circular DNA (cccDNA) containing the *Xho*I site (Figure 2B and b). The frameshift caused by the insertion is located within the presumed intron, 11 bp downstream of a potential splice donor site (Figure 1e). The splicing of this GT dinucleotide to an AG dinucleotide at codon 9 of ORF IV removes the mutation from mature mRNA and would explain why the frameshift is not deleterious. As a consequence of the splicing event an ORF III−ORF IV fusion protein will be synthesized (see Figure 1c and d; boxed amino acids: HLYS−ESPG).

cDNA fragment amplification of a spliced RNA

To prove that splicing of this mRNA occurs *in vivo*, we analysed the cDNA, obtained by a modified polymerase chain reaction using RNA of WDV infected wheat tissue as a template (Saiki *et al.*, 1985). This very sensitive technique allowed the analysis of the low abundant minus-strand RNAs without cloning their cDNAs. Two synthetic primers which bracket the position of the putative intron were used for the amplification reaction. The analysis of the products (Figure 3) showed a fragment of 298 bp which corresponds to an unprocessed mRNA (or to residual single-stranded viral DNA still present in the RNA preparations) and a smaller fragment, 212 bp in size, which presumably results from a spliced mRNA since the intron has a size of 86 bp (Figure 3, lanes 3, 4 and 6). It has exactly the same length as a fragment amplified from a single-stranded WDV template cloned in M13mp11, from which the potential intron sequence (see Figures 1e and 2C) was removed by oligonucleotide directed mutagenesis (Figure 3, lane 2). The ratio between the amplification products of the processed versus the unprocessed RNA varied in different amplification reactions (cf. lanes 3 and 6 of Figure 3).

Replication of the 'cDNA' variant of WDV

To test whether the ORF III−IV fusion protein encoded by the WDVΔi mutant (Figure 2C) is by itself sufficient to support the replication of WDV, DNA of this mutant was used to transfect protoplasts in the same way as described above, and replicative supercoiled DNA was detected at 8 days after transfection (Figure 2c).

Discussion

A detailed comparison of the sequences surrounding the intron reveals that splice donor and acceptor sites which optimally fit the consensus sequences (Green, 1986), also occur at equivalent positions on the genomes of all other geminiviruses of monocot plants sequenced to date, including the different isolates of maize streak virus, digitaria streak virus and chloris striate mosaic virus (Figure 1e, bold face).

A lariat sequence (CTGAC) is positioned ahead of the splice acceptor site at a distance characteristic for the branch point (Green, 1986). The excision of this particular intron sequence results in a fusion product of ORF III and ORF IV with maximal amino acid homology to the 40 kd protein of geminiviruses of dicot plants.

The 'intronic' and C-terminal amino acid sequences encoded by ORF III are also conserved among the geminiviruses of monocot plants (see Figure 1c, amino acids in *italics*), suggesting that this part of ORF III also encodes protein. However, DNA replication of WDV *per se* requires expression of the fused 41 kd ORF III−IV polypeptide only. It is therefore tempting to speculate that the expression of two different polypeptides, an individual ORF III protein which might be derived from unspliced mRNA and a fusion product derived from processed mRNA, might be important

Fig. 1. Comparison of geminivirus genomes. (**a**) ORFs on the genome of wheat dwarf virus (WDV) and on DNA A, the replication proficient half of the genome of TGMV, a geminivirus of dicot plants. The protein sequences of the 30 kd and the 17 kd ORFs of WDV are highly homologous to the continuous 40 kd ORF of TGMV. The position of the intron is shaded, its borders are marked by asterisks. ORFs of WDV are numbered I−IV. In the 40 kd ORF of TGMV the end of the homology with the 30 kd ORF, which coincides with the beginning of the homology with the 17 kd ORF of WDV is indicated by dots. (The orientation of the geminivirus genomes is such that the first nucleotide of the sequence motif 'TAATATTA' is defined as base 1 of the motif. It is present in the loop of a stem−loop structure and is conserved in the intergenic region of all known geminiviruses.) (**b**) Schematic alignment (bar code type) of the corresponding ORFs of four geminiviruses of monocot plants (**M**) to the 40 kd ORFs of four geminiviruses of dicot plants (**D**). Only identical amino acids between all geminiviruses are indicated by vertical bars. The overall conserved amino acid homology is about 36%. To obtain this match the 17 kd ORF was moved by the extent of the intron (shaded) in the amino-terminal direction. By doing so, the splice sites (*) on the DNA sequence appear next to each other. (**c**) Protein sequences encoded around the position of the intron. Identical amino acids are shown in negative printing, they correspond to the schematic bar code pattern displayed in part **b** of the figure. The dicot geminiviruses (**D**) are cassava latent virus (CLV), tomato golden mosaic virus (TGMV), bean golden mosaic virus (BGMV), beet curly top virus (BCTV). Monocot geminiviruses (**M**) are wheat dwarf virus (WDV), maize streak virus (MSV), digitaria streak virus (DSV) and chloris striate mosaic virus (CSMV). Aminoacids of the 30 kd and 17 kd ORFs, which become joined in the fusion protein are boxed. 'Intronic' amino acids are in *italics* and are displayed shaded (for comparison see **part a** of **Figure 1**, showing the position of the intron prior to the alignment for maximal homology). Amino acids in the C-terminal part of the 30/31 kd ORFs are also in *italics*. (**d,e**) Blow up of the intron region. (**d**) Amino acids around the intron region. ORFs III and IV of WDV are shown in their original overlap. Amino acids encoded by the intron sequence are shaded. The altered C-terminal amino acid sequence resulting from the frameshift in the WDV FSi mutant is shown below the sequence of the wild type. (**e**) DNA sequence of the WDV intron and of the corresponding sequences of three other monocot geminiviruses (MSV, DSV, CSMV). The splice sites and the lariat acceptor sequences are displayed in bold. The two additional G residues inserted at position 1876 to generate the frameshift mutation of WDV FSi are indicated. The sequence including the GT and AG dinucleotides (86 bases) is removed in the deletion mutant WDVΔi, the 'cDNA' variant of WDV (**Figure 2C**).

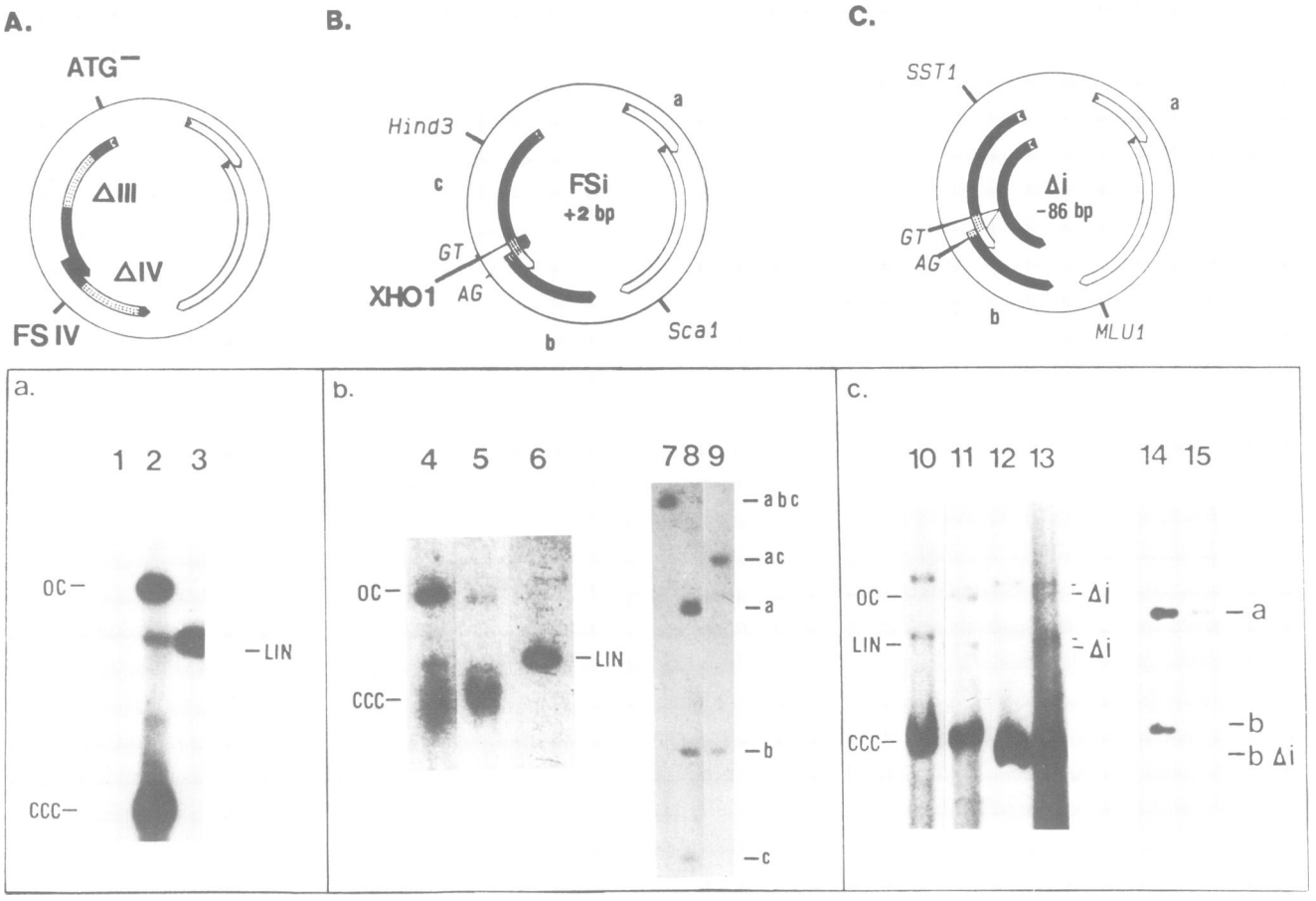


Fig. 2. Replication of WDV mutants in plant cells. (**A**) In the mutant (ATG⁻) four bases comprising the sticky ends of the *Nco*I site including the ATG codon of ORF III were deleted. The mutant (ΔIII) lacks 294 bp between a *Sst*I and a *Nru*I site. In the mutant ΔIV a 286 bp *Aat*II fragment is deleted. The frameshift mutant FS IV was generated by insertion of an 8 bp *Xho*I–*Nde*I adapter molecule into the unique *Nde*I site. (**B**) WDV FSi mutant: two additional guanine residues were introduced after base 11 of the intron to create a unique *Xho*I restriction site (see also **Figure 1e**). The resulting shift to a third frame and the premature termination of ORF III is represented by the small black arrow following the *Xho*I site. (**C**) The artificial 'cDNA' variant of WDV (Δi). The intron (86 bp) has been removed and ORFs III and IV are fused to generate an ORF with a coding capacity of 41 kd (inner black arrow). Stippled areas indicate the extent of the deletions (**A**) or the extent of the intron (**B,C**). Restriction sites that are relevant for the analysis are indicated, the fragments produced are labelled a, b and c. (**a**) Transfections with mutants of ORFs III and IV. About 20 μg of total DNA was loaded/lane, if supercoiled WDV molecules were purified, an equivalent amount was used. DNA of *T.monococcum*, after transfection with DNA of the ΔIV as an example of a replication deficient mutant (**lane 1**), no replicating DNA forms are detectable. Transfections with DNA of the ATG⁻, ΔIII, FS 75 IV mutants, as well as transfections with mixtures of these DNAs did not lead to detectable levels of replicating molecules (data not shown). **Lanes 2** and **3**: *T.monococcum* DNA after transfection with wild type WDV cloned via its unique *Hin*dIII site; **lane 2**: undigested; **lane 3**: digested by *Hin*dIII. (**b**) Replication of the frameshift mutant FSi. The WDV FSi DNA was cloned via the unqiue *Hin*dIII site within ORF III in M13mp11. Prior to transfection it was released from the vector by cleavage with *Hin*dIII. **Lane 4**: replicative WDV molecules isolated from naturally infected wheat tissue (*Triticum aestivum*, cv. *Drabant*) as a positive control; **lane 5**: *T.monococcum* DNA after transfection with FSi DNA, isolated 8 days post transfection; **lane 6**: same DNA as in **lane 5**, but cleaved with *Xho*I; **lane 7**: about 50 pg of *Hin*dIII treated input DNA of the mutant WDV FSi; **lane 8**: *Hin*dIII cleaved input DNA of the FSi mutant, but additionally digested by *Sca*I and *Xho*I to produce the fragments a, b and c (see **panel B** of **Figure 2**); **lane 9**: replicative DNA of WDV FSi DNA (same as in **lane 5**) digested by *Sca*I and *Xho*I. The *Hin*dIII site has become rejoined in the plant cells and a functional ORF III is reconstituted. At this time after transfection (8 days) no input DNA is detectable any more; instead only the joined fragment a+c is lit up. The fragments are generated from the covalently closed genome of WDV FSi, whose intact ccc molecules are shown in **lane 5**. (**c**) Replication of the intron deletion mutant WDVΔi. **Lane 10**: replicative forms of a slightly enlarged (2849 bp) WDV mutant. In this mutant the entire gene encoding the capsid protein (ORF III) is replaced by the neomycin phosphotransferase gene (WDVneo1). **Lane 11**: replicative forms of the WDVΔi mutant (2663 bp). **Lane 12**: replicative forms of wild-type WDV (2749 bp). **Lane 13**: DNAs from individual transfections with WDV wild-type DNA or WDVΔi DNA (isolated 8 days post transfection) were mixed and electrophoresed in the same lane to demonstrate the 86 bp difference in size (2749 bp versus 2663 bp); **lane 14**: replicative forms of WDV wild type (same as in **lane 12**), cut by *Sst*I and *Mlu*I to produce fragments a (1605 bp) and b (1144 bp). **Lane 15**: replicative forms of the mutant WDVΔi (same as in **lane 11**) cut by *Sst*I and *Mlu*I. The smaller fragment bΔi (1058 bp) lacking the 86 bp intron is lit up.

for the host specificity of monocot geminiviruses. This intron has been conserved during evolution. Its differential splicing might allow the expression of two proteins from one DNA sequence.

All geminiviruses of dicot plants including beet curly top virus (BCTV), a geminivirus with a monopartite genome, possess only the single continuous 40 kd ORF. We suggest that the expression of ORFs III and IV via a spliced mRNA is a general feature of all geminiviruses of graminaceous monocots and not a peculiarity of wheat dwarf virus. This geminivirus represents the first example among plant viruses the gene expression of which involves mRNA splicing.

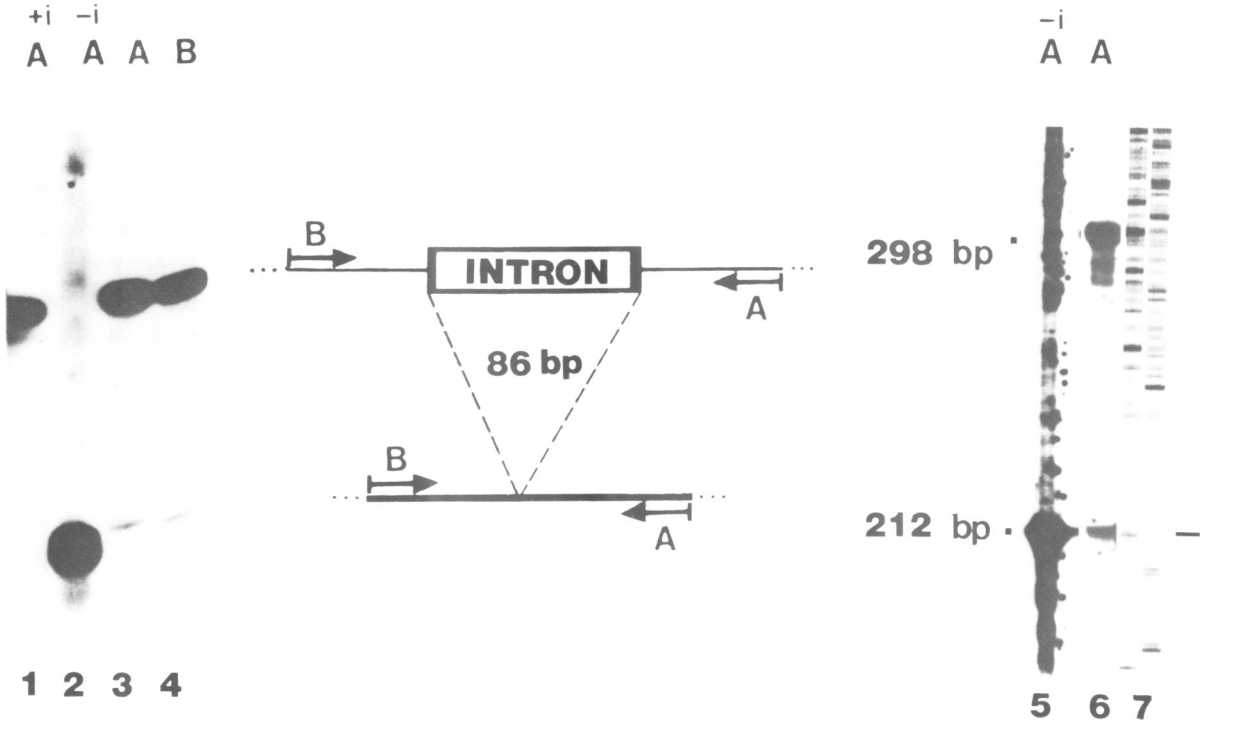


Fig. 3. Demonstration of a spliced minus-sense transcript by 'cDNA fragment amplification' (CFA). **Lanes 1** and **2**: polymerase chain reactions with ssDNA as controls. **Lane 1**: PCR with DNA of wild-type WDV, cloned in M13mp11 as a template. A single 298 bp fragment is generated. **Lane 2** and **5**: M13 template, but containing the WDVΔi mutation (see **Figure 2C**). A 212 bp fragment (298 bp−86 bp intron sequence) is generated. The fragments and the positions of primers A and B are displayed schematically in the panel between the corresponding bands on the autoradiographs (**upper line**: 298 bp fragment, **lower line**: 212 bp fragment). **Lanes 3, 4** and **6**: CFA of total RNA, isolated from WDV infected wheat plants; **lanes 3** and **6**: 5′ labelled primer A was added to the last amplification step; **lane 4**: 5′ labelled primer B was added to the last amplification step; **lane 7**: A and C tracks of a dideoxy sequence reaction.

Materials and methods

Generation of the mutants

The *Nco*I mutant (ATG$^-$) was generated by cleavage with *Nco*I, treatment with mung bean nuclease and ligation. The (ΔIII) deletion mutant was generated by cleavage with *Sst*I and *Nru*I, treatment of the ends with Klenow fragment of polymerase I and subsequent ligation. In the ΔIV mutant a 286 bp *Aat*II fragment was deleted by cleavage with *Aat*II and ligation. The frameshift mutation (FSi) and the deletion of the intron (Δi) were generated by oligonucleotide directed mutagenesis using the gapped duplex method and M13mp11 as vector (Kramer *et al.*, 1984). The correctness of these mutations was confirmed by DNA sequencing.

Transfection of plant protoplasts

10 μg of mutant WDV DNA, cloned in phage M13mp11, was released from the vector by cleavage with *Hin*dIII and used to transfect protoplasts of *T.monococcum* suspension culture cells as described (Hein *et al.*, 1983; Lörz *et al.*, 1985).

Demonstration of WDV DNA by Southern blot hybridization

Total DNA of *T.monococcum* cells was isolated at 8 days post transfection of the protoplasts. After treatment with restriction endonucleases, aliquots were separated on 1% agarose gels, transferred to nylon membranes (Hybond, Amersham) and hybridized to a ^{32}P-labelled WDV specific probe.

cDNA fragment amplification (CFA)

The CFA reaction was carried out using the following primers: A: 5′-gtg-tgtccctagagaccttgcccagg-3′ (5′ end at sequence position 1715) and B: 5′-gac-ttcgagtacacggcacgcc-3′ (5′ end at sequence position 2013): synthesized on an Applied Biosystems model 380 B. Primer A was annealed to the minus-sense transcript and extended in 5′-direction by M-MLV reverse transcriptase (BRL). Primer B, which hybridizes to the cDNA 298 bases upstream of primer A, was extended towards the 5′ end of primer A by Taq polymerase (New England Biolabs). Double-stranded cDNA was amplified by repeated (15 times) primer extensions of A and B in a polymerase chain reaction (PCR) as described (Saiki *et al.*, 1988). 5′-^{32}P-Labelled primers A or B were added to the last amplification cycle. Amplified fragments were separated on denaturing 6% polyacrylamide gels and detected by autoradiography, or were run on 6% sequencing gels (Figure 3, lanes 5−7).

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References

Andersen,M.T., Richardson,K.A., Harbison,S.-A. and Morris,B.A.M. (1988) *Virology*, **164**, 443−449.
Craigen,W.J. and Caskey,C.T. (1987) *Cell*, **50**, 1−2.
Donson,J., Acotto,G.P., Boulton,M.I., Mullineaux,P.M. and Davies,J.W. (1987) *Virology*, **161**, 160−169.
Goodman,R.M. (1977) *Virology*, **83**, 171−179.
Goodman,R.M. (1981) In Kurstak,E. (ed.), *Handbook of Plant Virus Infections and Comparative Diagnosis*. Elsevier, Amsterdam, pp. 883−910.
Green,M.R. (1986) *Annu. Rev. Genet.*, **20**, 671−708.
Gronenborn,B. and Matzeit,V. In Vasil,I.K. and Schell,J. (eds), *Cell Culture and Somatic Cell Genetics of Plants*. Vol. **6**, Academic Press, New York, in press.
Hamilton,W.D.O., Stein,V.E., Buck,K.W. and Coutts,R.H.A. (1984) *EMBO J.*, **3**, 2197−2205.
Harrison,B.D., Barker,H., Bock,K., Guthrie,E.J., Meredith,G. and Atkinson,M. (1977) *Nature*, **270**, 760−762.
Harrison,B.D. (1985) *Annu. Rev. Phytopathol.*, **33**, 55−82.
Hein,T., Przewozny,T. and Schieder,O. (1983) *Theor. Appl. Genet.*, **64**, 119−122.
Howarth,A.J., Caton,J., Bossert,M. and Goodman,R.M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3572−3576.
Howell,S.H. (1984) *Nucleic Acids Res.*, **12**, 7359−7375.
Kramer,W., Drutsa,V., Jansen,H.W., Kramer,B., Pflugfelder,M. and Fritz,H.J. (1984) *Nucleic Acids Res.*, **12**, 9441−9456.

Lazarowitz,S.G. (1988) *Nucleic Acids Res.*, **16**, 229–249.
Lindsten,K., Lindsten,B., Abdelmoeti,M. and Juntti,N. (1980) *Proceedings of the 3rd Conference on Virus Diseases of Gramineae in Europe*. Rothamsted Experimental Station, Harpenden, Herts, UK, 27–32.
Lörz,H., Baker,B. and Schell,J. (1985) *Mol. Gen. Genet.*, **199**, 178–182.
MacDowell,S.W., Macdonald,H., Hamilton,W.D.O., Coutts,R.H.A. and Buck,K.W. (1985) *EMBO J.*, **4**, 2173–2180.
Morris-Krsinich,B.A.M., Mullineaux,P.M., Donson,J., Boulton,M.I., Markham,P.G., Short,M.N. and Davies,J.W. (1985) *Nucleic Acids Res.*, **13**, 7237–7256.
Mullineaux,P.M., Boulton,M.I. and Davies,J.W. (1984) *EMBO J.*, **3**, 3069–3079.
Rogers,S.G., Bisaro,D.M., Horsch,R.B., Fraley,R.T., Hoffmann,N.L., Brand,L., Elmer,J.S. and Lloyd,A.M. (1986) *Cell*, **45**, 593–600.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Ehrlich,H.A. (1985) *Science*, **239**, 487–491.
Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Ehrlich,H.A. and Arnheim,N. (1988) *Science*, **230**, 1350–1354.
Shepherd,R.J. and Lawson,R.H. (1981) In Kurstak,E. (ed), *Handbook of Plant Virus Infections and Comparative Diagnosis*. Elsevier, Amsterdam, pp. 848–878.
Stanley,J. and Gay,M.R. (1983) *Nature*, **301**, 260–262.
Stanley,J. (1985) *Adv. Virus Res.*, **30**, 139–177.
Stanley,J., Markham,P.G., Callis,R.J. and Pinner,M.S. (1986) *EMBO J.*, **5**, 1761–1767.
Townsend,R., Stanley,J., Curson,S.J. and Short,M.N. (1985) *EMBO J.*, **4**, 33–37.
Townsend,R., Watts,J. and Stanley,J. (1986) *Nucleic Acids Res.*, **14**, 1253–1265.
Vacke,J. (1972) *Vedecke Prace Vyz. Ustavu Rostl. Vyroby*, **17**, 151–162.

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