Genomics of the Severe Isolate of *Maize Chlorotic Dwarf Virus*

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Abstract

Chaouch R., Redinbaugh M.G., Marrakchi M., Hogenhout S.A. (2004): **Genomics of the severe isolate of *Maize chlorotic dwarf virus*.** Plant Protect. Sci., **40**: 113–119.

The complete sequences of the severe isolates of *Maize chlorotic dwarf virus* **(**MCDV-S) were obtained from cDNA clones of the viral RNA. The genome is 11 785 nucleotides (nt) in length and contains one large open reading frame between nt 455 and 10 825 that encodes a polyprotein of 3458 amino acids. Computer analysis revealed several conserved protein motifs similar to other plant picorna-like viruses. Comparison of the genome sequence of MCDV-S with that of the type strain of MCDV showed high similarities despite the difference in symptoms.

Keywords: plant picorna-like virus; sequence comparison; protein motif; *Waikavirus*

An economically devasting maize disease throughout the southeastern part of the United States, commonly known as *Maize chlorotic dwarf virus* (MCDV) is caused by a complex of strains of MCDV. It is considered to be the second major corn virus disease in the USA (Knoke & Louie 1981). Four distinct isolates/strains of MCDV have been described, based on symptoms, serology, and/or geographical location: MCDV-type (MCDV-T), MCDV-mild (MCDV-M1), MCDV-severe (MCDV-S), and the Tennessee isolate of MCDV (MCDV-TN) (Hunt *et al*. 1988; Gingery & Nault 1990; Reddick *et al*. 1997). Symptoms caused by MCDV-S are more pronounced than those of MCDV-T, which are more severe than those of MCDV-M1. However, plants co-infected with MCDV-M1 and MCDV-T in the greenhouse exhibit the extreme stunting and leaf tearing often seen in field infections (Gingery & Nault 1990).

Symptoms caused by the severe strain of this virus include severe stunting, leaf discoloration (reddening and yellowing), and leaf-tearing of maize (Bradfute *et al*. 1972; Gordon & Nault 1977). A consistent, diagnostic symptom of MCDV-S infection is chlorosis of the tertiary leaf veins (vein banding) (Gordon & Nault 1977; Pratt *et al*. 1994). Transmission is by the detocephaline leafhopper, *Graminella nigrifrons* (Forbes), in a semi-persistent manner (Gingery *et al*. 1981). Morphologically, MCDV virions are 30 nm diameter icosahedrons with a buoyant density of 1.507 g/ml (Gingery 1976). The genome consists of a single-stranded RNA molecule with an estimated molecular mass of 9.4 kDa (Gingery *et al*. 1981). These properties of MCDV resemble those of *rice tungro spherical virus* (RTSV) within the family of *Sequiviridae* (Shen *et al*. 1993). These two viruses and *anthriscus yellow virus* have been placed in the genus *Waikavirus* (Mayo *et al*. 1995).

In this paper, we present the nucleotide sequence of MCDV-S and the predicted coding region of the viral genome. The nucleotide and protein sequences of MCDV-S are compared with the published sequences of a type isolate of MCDV: MCDV-T (McMullen *et al*. 1996), MCDV-TN (Reddick *et al*. 1997) and other plant picorna-like viruses.

MATERIAL AND METHODS

The isolate of MCDV used in this study was obtained and isolated from infected maize plants (*Zea mays* L.) maintained in a greenhouse. Since MCDV-S is not mechanically transmitted, the virus was multiplied by serial transfer with the leafhopper *Graminella nigrifrons*. Leafhoppers were allowed a 24 h acquisition feeding period and a 48 h inoculation feeding period on Seneca Chief or OH-28 sweet corn seedlings. MCDV-S particles were then purified using the method of Gingery *et al*. (1978).

RNA extraction and cDNA synthesis. RNA was extracted from purified MCDV-S virions by phenol-chloroform, and precipitated with ethanol/sodium-acetate (Sambrook *et al*. 1989). The extracted RNA was resuspended in 10 µl of deionised water. The first strand cDNA of the full length genome was synthesised with a T (dT) primer by RT-PCR using the ThermoScript RT-PCR system according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA).

Rapid Amplification of cDNA Ends (RACE). The cDNA of the 5′ extreme end of MCDV-S was synthesised by reverse transcription coupled with polymerase chain reaction using the 5′ RACE kit from GIBCO-BRL. The 5′ RACE involved an RT-PCR using a MCDV-S Gene Specific Primer GSP1 (5′ CATTC TCTCTTCCGGAGCG 3′). The RT-reaction was treated with phenol, precipitated with ethanol, resuspended in 10 µl of deionised water (Sambrook *et al*. 1989), and poly (C) tailed by terminal transferase. The first PCR used a second GSP 2 (5′ CTAGCT AGAGCCTGACGAC 3′) with the Abridged Anchor Primer (AAP) (GIBCO-BRL), at low stringency annealing (40°C) followed by a second PCR using a nested GSP 3 (5′ TTACG-GCATG ACTTATAGC GCATTGCGACT 3′) with the Abridged Universal Amplification Primer (AUAP) (GIBCO-BRL). The 3′ end of the MCDV-S genome was resolved also from an RT-PCR product amplified with a NN poly(T) degenerate primer (5′ GGGAAGTCTAGATTTTTTTTTTTTTTTTT (AGCT) (AGCT)). PCR products were separated by gel electrophoresis in 1% agarose, and visualised after staining with ethidium bromide under UV light. Fragments were excised from the gel by Geneclean (Qiagen) and ligated into pGEM-T Easy vector.

Sequencing and sequence comparison. Nucleic acid sequences were determined by primer walking, using a Perkin-Elmer ABI model 377 Prism DNA sequencing machine using the ABI BigDye Terminator Reaction kit (Applied Biosystems, Foster City, CA). For each PCR product, sequences of both strands for two independent clones were determined. The MCDV-S genome sequence was determined from overlapping PCR products synthesised with a proof reading Platinum *Taq* polymerase High Fidelity (Invitrogen) and MCDV-T specific primers were used (Table 1). PCR fragments were cloned into the pGEM-T- easy vector using competent *Escherichia coli* strain DH5 alfa (STRATAGENE) by standard procedures (Sambrook *et al*. 1989).

The obtained sequences were initially base called using MacPHRED-MacPHRAP package software and edited and analysed using the "sequencher4.1" assembly package software of Applied Biosystems. Further analysis and sequence comparisons were performed with AssemblyLIGN™, LRP's DNA sequence assembly program, DNA strider 1.2 software, Amplify 1.2 software. Multialignments were then done with the clustal × program (Thompson *et al*. 1997) after bootstrapping in 1000 replicates, and Phylogenies were viewed by the njplot program.

RESULTS

Characterisation of the MCDV-S genome

The MCDV-S had earlier been identified by symptoms, serological tests and transmission studies. We now determined the complete nucleotide sequences of MCDV-S RNA. The MCDV-S genome was 11 785 nucleotides long excluding the 3′ poly (A) region (Gen-Bank accession AY362551).

The MCDV-S sequence was constructed from multiple clones (Figure 1) spanning the entire genome. All clones (S1 to S6) were sequenced in both directions. The sequence of the 5′ terminus was resolved from 5′ RACE amplicons. The 3′ end sequence was obtained from a RT-PCR product amplified with a T (dT) degenerate primer.

Computer analysis of the MCDV-S sequence revealed three open reading frames (ORFs) in

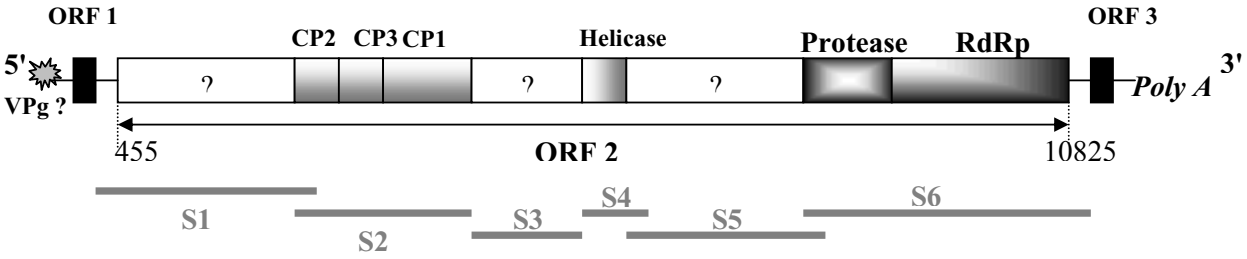

Figure 1. Genome organisation of MCDV-S. Boxes represent open reading frames (ORFs) with encoded proteins designated above: Coat proteins (CP1, CP2 and CP3): Helicase, Proteinase, Polymerase (RdRp). White boxes with question marks are not yet characterised as well as the virus encoded protein (VPg). Segments S1 to S6 represent overlapping PCR clones used for sequencing MCDV-S genome

the positive-sense orientation. The largest ORF starts at the AUG codon located at nucleotide 455, and ends with an UAA codon at nucleotide 10825 (Figure 1).

The large ORF would encode a 3458 amino acids protein with a calculated molecular mass of 389 kDa. Two small ORFs were identified at the 5′ end, at nucleotides 85 to 180, and at the 3′ end, at nucleotides 11139 to 11343 which could encode small proteins. The unique feature common to all picornaviruses is the presence of a long 5′ untranslated region (UTR) 600 to 1400 bp long, with several AUG translation start codons preceding the actual initiation AUG. The 5′ non-coding sequence contained extensive secondary structure based on analysis by Mulfold (Zucker 1989). The 5′ UTR folds into several stem loop secondary structures named the internal ribosome entry site (IRES). The IRES directs the binding of the host 80S ribosome to the main translation initiation start codon. The IRES 3′ border is characterised by the presence of a pyrimidine rich tract (Y_n) separated from the downstream AUG by a spacer (X_m) region of 15–20 nucleotides (Wimmer *et al*. 1993; Druka *et al*. 1996; Hull 1996).

Conserved protein motifs

The 389 kDa polyprotein encoded by the largest ORF contains three consensus motifs of the RNA-dependent RNA polymerase (RdRp) of positive-strand RNA viruses (Koonin & Dolja 1993). The (RdRp) or replicase region was found at the most C-terminal region of the MCDV-S polyprotein (Figure 1). Conserved domains analogous to the RdRp of picorna-like viruses were identified in MCDV-S. The domain A motif (DYSxFDGxxxP) was found at amino acids 3143-3154 (**DYS**K**FDG**IGD**P**); domain B (SgxxxTxxxNS) at 3204-3215 (**SG**FAM**T**VIF**NS**); and domain C (YGDD) located at amino acids 3252-3256 (Bruenn 1991; Poch *et al*. 1989).

The motif **GxxGxGKS** (Blinov *et al*. 1998) was found, representing the helicase proteins of other plant picorna-like viruses. This region was located between amino acids 1755 and 1818 (**G**AP**G**V**GKS**) and was similar to the helicase amino acid sequence in MCDV-T polyprotein.

The polyprotein of MCDV-S also contains a region with similarities to picorna-like viral proteinases (Turnball-Ross *et al*. 1992; Shen *et al*. 1993). The catalytic triad of histidine, aspartate and cysteine H, E/D and C (Gorbalenya *et al*. 1989a) conserved in cysteine proteases (3C-PRO) (Gorbalenya *et al*. 1989b; Dessens & Lomonossoff 1991; Margis & Pinck 1992). Interestingly the amino acid sequence between 2613-2820 shows similarity to the 3C protease. It was not surprising that the MCDV-T and MCDV-S coat proteins were cleaved as Q/V for CP2, Q/L for CP3 and Q/M for CP1, respectively, confirming that the specificity of cleavage by 3C proteases is the peptide bond between glutamine (Q) and amino acid residues with small side chains (Allaire *et al*. 1994; Druka *et al*. 1996). On the other hand, the 5′ linked VPg protein (virus encoded protein) usually present in animal picornaviruses has not been confirmed yet (pending confirmation of results).

Sequence comparison

Complete nucleotide and polyprotein sequences of MCDV-S and MCDV-T were aligned using Clustal X (Thompson *et al*. 1997) and compared in Table 1.

Table 1. Name, sequence, and location of primers used for amplification of MCDV-S genome regions. Primers were designed on the bases of the published sequence of MCDV-T (GenBank accession number I28269)

Primer	Primer sequence (5′ – 3′)	Location
S1-for	ATGATGCAGACAAACAACAAC	455–475
S1-rev	TTGCTTCACAACTGGTCT	2512–2495
S2-rev	aaatgtatccacagaagccaatttttcc	4804–4831
S2-for	ctcgagtgaaggcaagaccagttgtg	2481–2504
S3-for	AAGGTGCTTTCGGAACGC	4637–4654
S3-rev	CTGGAGCAACTTATAGGT	5596–5579
S4-for	agtggttttggtggtgatgagcatct	5513–5538
S4-rev	actgtagttcatgttcatgagatcttc	6283–6257
S5-for	CAAGTGCTTGATGGAGGTAG	6182–6201
S5-rev	TTGCTTGATGGCTCTT	7993–7978
S6-for	tgtaacttacgatgacgaacccagc	7909–7933
S6-rev	gctaccccactttatctatagtg	11761–11784

The two genomes were found to be closely related at both the nucleotide and protein level. However, the MCDV-S genome lacks one AUG in the 5′ non-coding region relative to MCDV-T in which the A at nucleotide position 319 was a G. Furthermore, a BLAST search (Devereux *et al*. 1984) revealed that MCDV-S was also closely related to MCDV-TN with 68.2% and to RTSV RNA with 47.5% identity. The amino acid sequence comparison of each motif was established between MCDV-S, MCDV-T , MCDV-TN and *Rice tungro spherical virus* (RTSV), *Parsnip yellow fleck virus* (PYFV), and the two plant picorna-like viruses *cow pea mosaic virus* (CPMV) and *Tobacco black ring virus* (TBRV) as shown in Table 2.

DISCUSSION

The genome organisation of MCDV-S resembles that of plant picorna-viruses in the *Sequiviridae* family. The MCDV-S genome has a positive-sense, ssRNA genome and is 11,785 nt in length. Considerable difficulties were encountered with sequencing the 5′ untranslated region of the genome. We considered that these problems were due to the secondary structure of the RNA. These were overcome by amplifying the 5′ region using the 5′ amplification kit and cloning it into pGEMT-easy system (Promega). A computer analysis of the nucleotide sequence of the MCDV-S genome

Table 2. Amino acids sequence of MCDV-S compared to other plant picorna-like viruses. Multiple alignments of amino acid sequence were obtained with CLUSTAL X (Thompson *et al*. 1997)

Virus	Whole genome (%)	Helicase (%)	Virus-protease (%)	RdRp (%)
MCDV-T	99.3	99.3	99.5	99.3
MCDV-TN	68.2	74	81	62
RTSV	47.5	40	67	39
PYFV	20	18	40	23
CPMV	–	33	–	31
TBRV	–	–	–	25

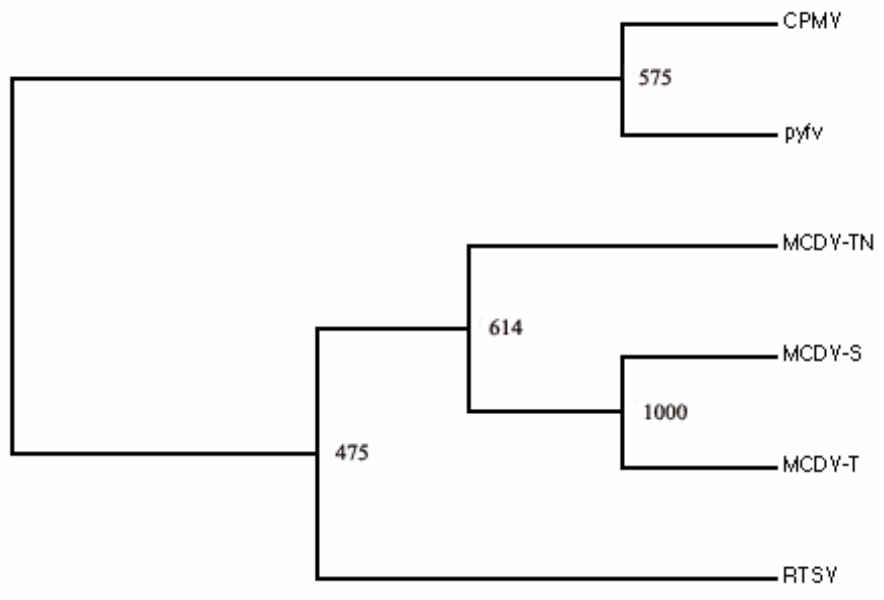

Figure 2. Cladogram was constructed by the neighbor-joining method (Saitou & Nei 1987) and the statistical significance of branch order was estimated by performing 1000 replications of bootstrap resampling of the original alignment with CLUSTAL X

revealed that the deduced amino acid sequence of ORF 2 contained the core motifs of the picornavirus 2C RNA helicase, 3C cysteine protease and 3D RNA-dependent RNA polymerase (RdRp). These motifs are also conserved in the genomes of viruses in the families *Comoviridae* and *Caliciviridae* (Koonin & Dolja 1993). The predicted amino acid sequences around these motifs were aligned with those of the insect picorna-like viruses, MCDV-T, MCDV-TN, RTSV, PYFV, CPMV and TBRV. Table 1 shows similarities in the RNA NTP binding site of these viruses. The consensus sequence for RNA helicase, GX% GK (Gorbalenya *et al*. 1989a, b), which is proposed to be responsible for nucleotide binding, was identified. The amino acid sequence 2613–2820 shows similarity to the 3C protease of mammalian picornaviruses, insect picorna-like viruses, and comoviruses. The cysteine protease motif GXCG was found. (Koonin & Dolja 1993). The conserved motifs of RdRp of positive-strand RNA viruses (Koonin 1991) were found in the C-terminal region of the putative ORF 2 product of MCDV-S (Figure 1). We analysed phylogenetic relationships between MCDV-S and the species in the *Sequiviridae* and other picorna-like plant viruses for amino acid sequences (Table 2). As shown in Figure 2, the phylogenetic tree places MCDV-S into a cluster with MCDV-T, MCDV-TN and RTSV.

We propose that much of the divergence among strains of MCDV may be explained by drift in which genetic isolation facilitates accumulation and fixation of neutral mutations. The duration of isolation then accounts for the amount of divergence between strains. However, selection also is an important factor. Clearly, much of the MCDV genome is constrained by negative selection such that certain regions of the genome retain high sequence identity (data not shown). The GDD polymerase motif represents a well known example of sequence conservation likely constrained by negative selection. Drift and selection are not mutually exclusive forces and may occur concurrently. Thus, it is the combined effects of both forces acting simultaneously on different regions of the genome that ultimately determine the extent of divergence within a viral species. In picornaviruses, RNA translation is dependent on IRES, and the introduction of single nucleotide mutations in this region changes symptom severity and phenotype of a picornavirus (Jackson & Kaminski 1995; Belsham & Sonenberg 1996; Rijnbrand *et al*. 1996; Pilipenko *et al*. 1999). We, therefore, propose that the 3-nucleotide differences in the IRES of MCDV-T and MCDV-S may be responsible for the differences in symptom severity between MCDV-T and MCDV-S.

Accession number

The nucleotide sequence reported in this paper has been deposited with the DDBJ/Gen-Bank/EMBL databases with the accession number AY362551.

Acknowledgements: The authors would like to thank Dr. Roy Gingery for helpful discussions, Pr Mohamed Marrakchi for his constant support and Kristen Willie for excellent technical assistance.

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Received for publication May 3, 2004
Accepted after corrections September 3, 2004

Souhrn

Chaouch R., Redinbaugh M. G., Marrakchi M., Hogenhout S.A. (2004): **Genomové sekvence několika izolátů *Maize chlorotic dwarf virus*.** Plant Protect. Sci., **40**: 113–119.

Byly získany kompletní sekvence několika izolatů *Maize chlorotic dwarf virus* (MCDV-S). Genom tohoto viru má délku 11 785 nukleotidů (nt) a obsahuje široký otevřený čtecí rámec mezi 455 a 10 825 nt, v rámci kterého je kódován polyprotein mající 3458 aminokyselin. Počítačová analýza odhalila několik konzervativních motivů u tohoto proteinu, jež jsou podobné jiným rostlinným virům příbuzným pikornavirům. Porovnání genomové sekvence MCDV-S se sekvencí typového kmene MCDV ukázalo značnou podobnost nehledě na rozdíly v symptomech.

Klíčová slova: rostlinný virus příbuzný pikornavirům; porovnání sekvencí; motif proteinu; *Waikavirus*

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