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Accumulation of *Maize chlorotic dwarf virus* proteins in its plant host and leafhopper vector

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Abstract

The genome of *Maize chlorotic dwarf virus* (MCDV; genus *Waikavirus*; family *Sequiviridae*) consists of a monopartite positive-sense RNA genome encoding a single large polyprotein. Antibodies were produced to His-fusions of three undefined regions of the MCDV polyprotein: the N-terminus of the polyprotein (R78), a region between coat proteins (CPs) and the nucleotide-binding site (NBS) (R37), and a region between the NBS and a 3C-like protease (R69). The R78 antibodies react with proteins of 50 kDa (P50), 35 kDa (P35), and 25 kDa (P25) in virus preparations, and with P35 in plant extracts. In extracts of the leafhopper vector *Graminella nigrifrons* fed on MCDV-infected plants, the R78 antibodies reacted with P25 but not with P50 and P35. The R69 antibodies bound proteins of approximately 36 kDa (P36), 30 kDa (P30), and 26 kDa (P26) in virus preparations, and P36 and P26 in plant extracts. Antibodies to R37 reacted with a 26-kDa protein in purified virus preparations, but not in plant extracts. Neither the R69 nor the R37 antibodies bound any proteins in *G. nigrifrons.* Thus, in addition to the three CPs, cysteine protease and RNA-dependent RNA polymerase, the MCDV polyprotein is apparently post-transitionally cleaved into P50, P35, P25, P36, P30, and P26.
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Introduction

Maize chlorotic dwarf virus (MCDV) is prevalent in the southeastern US within the region overlapping the distributions of its vector, the black-faced leafhopper (*Graminella nigrifrons* Forbes), and its overwintering host, the perennial weed Johnson grass (*Sorghum halepense* (L.) Pers.) (Gordon and Nault, 1977; Gordon et al., 1981; Lopes et al., 1994). MCDV causes severe stunting, leaf discoloration (reddening and yellowing), and leaf tearing in maize (Bradfute et al., 1972; Gordon and Nault, 1977). The diagnostic symptom of MCDV infection is chlorosis of the tertiary leaf veins (vein banding) (Gordon and Nault, 1977; Pratt et al., 1994). MCDV belongs to the genus *Waikavirus* of the family *Sequiviridae*. Other sequiviruses include the leafhopper-transmitted *Rice tungro spherical virus* (RTSV) in the genus *Waikavirus* and the aphid-transmitted *Anthriscus yellows virus* (AYV) and *Parsnip yellow fleck virus* (PYFV) in the genus *Sequivirus* (Hull, 2002).

MCDV infection is restricted to phloem-associated cells in maize, particularly the vascular parenchyma, companion cells, and immature sieve tubes (Ammar et al., 1993). The virus is transmitted by the leafhopper *G. nigrifrons* in a semi-persistent manner, that is, leafhoppers acquire MCDV from infected plants and can inoculate healthy plants within a few hours of feeding, and lose their ability to transmit the virus within 2–4 days after virus acquisition or after molting (Nault et al., 1973). *G. nigrifrons* is not capable of transmitting purified virus particles alone but apparently requires helper

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component (HC) protein(s) synthesized in plants during MCDV infection (Creamer et al., 1993; Hunt et al., 1988). Transmission electron microscopy showed that MCDV particles were embedded in a densely stained matrix in both infected plant cells and in leafhopper foreguts (Ammar and Nault, 1991; Ammar et al., 1993). Matrix and virus particles were attached to the cuticular lining of the maxillary food canal, precibarium, cibarium, and pharynx in leafhoppers fed on MCDV-infected plants.

Four distinct isolates or strains of MCDV have been described based on symptoms, serology, or geographical location: MCDV-type (MCDV-T), MCDV-mild (MCDV-M1), MCDV-severe (MCDV-S; previously called MCDV White Stripe or MCDV-WS), and the Tennessee isolate of MCDV (MCDV-TN) (Gingery and Nault, 1990; Hunt et al., 1988; Reddick et al., 1997). MCDV-S symptoms are more pronounced than those of MCDV-T, which in turn are more severe than those of MCDV-M1. However, plants co-infected with MCDV-M1 and MCDV-T in the greenhouse exhibit extreme stunting and leaf tearing often seen in field infections (Gingery and Nault, 1990).

MCDV particles are isometric and composed of three coat proteins (CPs), which are the only structural proteins of mature MCDV particles detected so far. The MCDV-S and MCDV-T CPs have calculated weights of 31 kDa (CP1), 23 kDa (CP2), and 22 kDa (CP3) on one-dimensional SDS-PAGE, and antisera raised against MCDV-T cross-react strongly with MCDV-S (Gingery and Nault, 1990). In contrast, on SDS-PAGE, CP1 and CP2 of MCDV-M1 are larger than those of MCDV-T, and MCDV-T antisera cross-react weakly with MCDV-M1 (Gingery and Nault, 1990). Thus, MCDV-T and MCDV-S may be considered isolates of the same virus strain, while MCDV-M1 is a distinct strain.

The genomes of MCDV-T and MCDV-TN have been sequenced (McMullen et al., 1996; Reddick et al., 1997). Both genomes are monopartite single-stranded positive-sense RNA molecules of about 11.8 kb encoding a 3475 amino acid open reading frame (ORF) that is translated into a single polyprotein posttranslationally cleaved into an unknown number of smaller proteins. The positions of the three coat proteins on the MCDV polyprotein are known (McMullen et al., 1996; Reddick et al., 1997). Further, based on motifs conserved among picornaviruses, a cysteine protease and an RNA-dependent RNA polymerase were identified in the MCDV polyprotein sequence. However, three regions of the MCDV polyprotein do not have similarity to sequences currently in GenBank, and the sizes of cleavage products or functions of proteins encoded by these regions have not been examined. These regions are the 686 amino acids at the N-terminus of the polyprotein adjacent to CP2, approximately 400 amino acids between the polyprotein CP1 and the NTP binding site (NBS), and approximately 800 amino acids between the polyprotein NBS and cysteine protease.

The objectives of this study were to sequence the MCDV-S genome, to determine the sizes of cleavage products of the three uncharacterized regions of the MCDV-S polyprotein, and to investigate whether these products can be detected in MCDV-S-infected plants, purified virus preparations, and viruliferous *G. nigrifrons*.

Results

Detection of MCDV structural proteins

MCDV particles are composed of three coat proteins that are present in equal amounts in virus particles. As expected, Coomassie brilliant blue-stained gels of MCDV particles reveal three structural proteins of similar intensities (Fig. 1A). On Western blots, antibodies raised against purified MCDV-T particles reacted strongly with the two larger MCDV-S and MCDV-T proteins (CP1 and CP2), but not with the smallest one (CP3) (Fig. 1B, lanes 1 and 2). The lack of reaction of MCDV-T antibodies with CP3 suggests that (i) CP3 is a weak antigen; (ii) CP3 is hidden inside MCDV particles, and hence, is unavailable for raising antibodies; or (iii) antibodies are produced to discontinuous epitopes of MCDV CP3 and do not react to CP3 once it has been denatured by SDS-PAGE. MCDV-T antibodies reacted weakly with CP1 of MCDV-M1 and did not react with the CPs of RTSV (Fig. 1B, lanes 3 and 4). This result confirms findings reported elsewhere that MCDV-T antibodies cross-react strongly with MCDV-S but weakly with MCDV-M1 (Gingery and Nault, 1990). The MCDV-T antibodies detected an additional protein that was slightly larger than CP1 in MCDV-S virions, but not in MCDV-T, MCDV-M1, and RTSV virions (Fig. 1B, lanes 1 and 2). This MCDV-S protein may be modified CP1 or another protein attached to virus particles.

The MCDV-S genome sequence

Sequence analysis of the MCDV-S genome would allow comparison with the published sequences of MCDV-TN

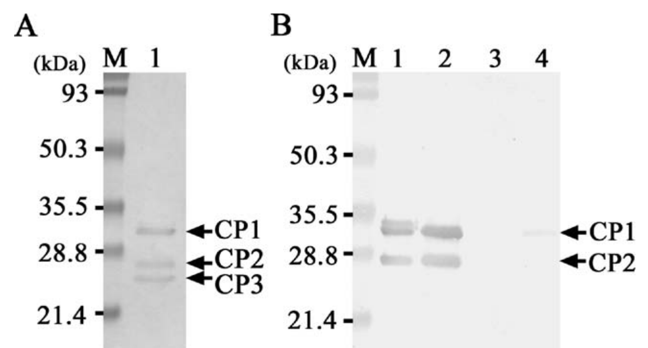

Fig. 1. Characterization of MCDV virion proteins. (A) Coomassie brilliant blue-stained SDS-PAGE gel of MCDV-S virions; (B) Western blot of SDS-PAGE gel of proteins from 3.75 μg MCDV-S (lane 1), MCDV-T (lane 2), RTSV (lane 3), and MCDV-M1 (lane 4) virion probed with MCDV-T antibodies. Molecular weights (kDa) from marker lanes (M) are shown at the left of the gels, and the positions of viral proteins are denoted with arrows.

(Reddick et al., 1997), MCDV-T (McMullen et al., 1996), and MCDV-M1 (Gingery et al., unpublished), and could provide insight into the molecular basis underlying the distinction in symptoms between the MCDV-T and -S strains. The MCDV-S genome was 11 785 nucleotides long excluding the 3′ poly(A) region (GenBank accession AY362551). The genome encoded a large ORF on the plus strand. Similar to other waikaviruses and sequiviruses, this ORF encodes a polyprotein beginning at an AUG codon (nt 455–457) and ending at a UAA codon (nt 10 828–10 830). The deduced protein sequence of this ORF is 3457 amino acids long and has a calculated molecular mass of 389 kDa. Three additional small ORFs were identified: one at the 5′ end (nt 85–180) and two at the 3′ end (nt 10 912–11 019 and 11 139–11 339).

MCDV-S is more similar to MCDV-T (99.3% identity) than to MCDV-TN (59.3% identity), RTSV (47.5% identity), or PYFV (35.4%) (Table 1). The MCDV-S and MCDV-T genome sequences have 81 nucleotide differences (Table 1). Of these, three are located upstream and 11 downstream of the large polyprotein ORF (Fig. 2). Similar to other picornaviruses (Rijnbrand et al., 1996), the 5′ noncoding regions of MCDV-T and MCDV-S genomic RNA have several start codons (11 and 10, respectively), preceding the putative start codon of the polyprotein ORF (data not shown). MCDV-S lacks the last AUG upstream of the polyprotein ORF start codon relative to MCDV-T.

In the polyprotein coding sequence of MCDV-T and MCDV-S, 18 of the 67 nucleotide differences resulted in amino acid changes. Thus, MCDV-S was 99.5% identical with the polyprotein sequence of MCDV-T. Of these 18 differences, 8 were substitutions with similar amino acids and 10 were amino acids of different polarity. The amino acid at position 1362 in the CP1 region of the polyprotein was found to be a leucine in MCDV-S and a serine in MCDV-T. This amino acid substitution is located immediately after the putative glycosylation site "NKSN" (Prosite at http://us.expasy.org/prosite/).

Regions of the MCDV-S polyprotein with similarity to the characterized CPs of MCDV-TN (Reddick et al., 1997) and MCDV-T (McMullen et al., 1996) are indicated in Fig. 2. The three MCDV CPs are located toward the N-terminus of the polyprotein. N-terminal sequences for CP3 and CP1 were experimentally determined for MCDV-T and MCDV-TN (McMullen et al., 1996; Reddick et al., 1997). Sequences identical to the MCDV-T CP3 and CP1 and similar to the MCDV-TN 3C-like protease cleavage sites were detected in the MCDV-S polyprotein between amino acids 896 and 897 for CP3, and 1098 and 1099 for CP1. The cleavage site surrounding the putative CP2 N-terminus at 687 in MCDV-S and MCDV-T is PVVKQ/SGTIM and compares with the experimentally confirmed cleavage site RVEKQ/SGTNI in MCDV-TN (Reddick et al., 1997). The sequence of the putative C-terminal cleavage site of MCDV-TN is NAVAQ/METIN (Reddick et al., 1997), and a similar sequence NATAQ/MDFDR with amino acid 1373 (Q) as the last amino acid of CP1 was detected in both MCDV-S and MCDV-T. Cleavage at these sites would produce coat proteins of 31 kDa for CP1, 23 kDa for CP2, and 22 kDa for CP3 (Fig. 2). A region with similarity to the 3C-like protease begins at amino acid 2613 and is adjacent to the RNA-dependent RNA polymerase (RdRp) located at the C-terminal end of the MCDV-S polyprotein. The 3C-like protease and RdRp of MCDV-S have 100% similarity to those of MCDV-T and 83.9% similarity to those of MCDV-TN. Finally, the protein region from amino acid 1755–1818 contains the conserved NBS in MCDV-S.

MCDV-S was used for further analysis of the three uncharacterized regions of the waikavirus polyprotein. These are the N-terminal region with a calculated molecular weight of 78 kDa (R78), the region between CP1 and NBS with a calculated molecular weight of 37 kDa (R37), and the region between NBS and 3C-like protease with a calculated molecular weight of 69 kDa (R69) (Fig. 2). BLASTP analysis of the deduced protein sequences and blastn analysis of the corresponding nucleotide sequences for these three regions against the GenBank nonredundant (nr) database did not result in significant hits other than corresponding sequences of other members of the family *Sequiviridae* (data not shown).

Production of synthetic proteins corresponding to three segments of the MCDV-S polyprotein

Three primer pairs were developed to amplify uncharacterized genome segments corresponding to MCDV-S polyprotein residues 1–686 (R78), 1377–1714 (R37), and 1911–2513 (R69) (Fig. 2 and Table 2). Amplified products were cloned into the pTrcHis B plasmid and expressed in *E. coli*.

Table 1
Nucleotide and amino acid identities and similarities among waikaviruses

Virus (Accession no.)		MCDV-T (I28269)[a]	MCDV-TN (20162543)	RTSV (9627950)	PYFV (20177429)
Nucleotides	Identity	11 704/11 785[b] (99.3%)	6992/11 785 (59.3%)	5594/11 785 (47.5%)	4180/11 785 (35.4%)
Amino acids	Identity	3439/3457 (99.5%)	2045/3457 (59%)	1183/3457 (34.2%)	688/3457 (20%)
	Similarity	8/3457 (0.2%)	510/3457 (14.8%)	599/3457 (17.3%)	482/3457 (14%)

MCDV-T, type strain of MCDV; MCDV-TN, Tennessee strain of MCDV; RTSV, *Rice tungro spherical virus* (genus *Waikavirus*; *Sequiviridae*); PYFV, *Parsnip yellows fleck virus* (genus *Sequivirus*, *Sequiviridae*).

[a] The GenBank accession number for the sequence used in the analysis.

[b] Number of nucleotide or amino acid residues in MCDV-S (GenBank accession AY362551) that are identical or similar to the above virus/total number of residues in MCDV-S.

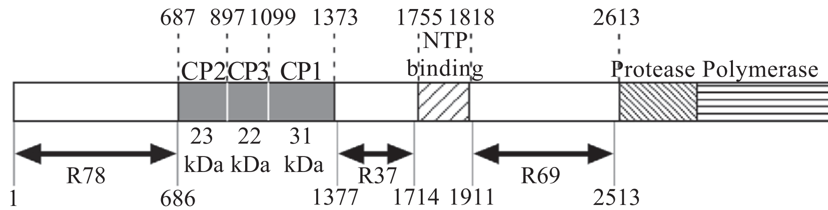

Fig. 2. MCDV-S polyprotein. The 11 785-bp RNA genome of MCDV-S encodes a 389-kDa polyprotein that is cleaved into several smaller proteins by virus-encoded or plant proteases. In addition to the three coat proteins (CP), regions with similarity to a putative nucleotide binding site (NBS), a 3C-like protease with a cysteine active site, and an RNA-dependent RNA polymerase are indicated. Three regions for which function is unknown, indicated as R78, R37, and R69, were selected for further study. The first and last amino acids of R78, R37, and R69 are indicated below the figure, and the first and last amino acids of coat proteins, NBS, and protease are marked with dashed lines above the figure.

The resulting His-fusion proteins were affinity purified, subjected to SDS-PAGE, and probed with anti-His polyclonal antiserum. The antiserum reacted with a fusion protein of the expected size in *E. coli* extracts (data not shown).

MCDV-S polyprotein products in purified virus

Processed viral proteins associated with the R78, R37, and R69 regions of the MCDV-S polyprotein were investigated in two virus preparations, purified virions and partially purified virions (see Materials and methods for purification details). Western blots of size-separated proteins were incubated with MCDV-T, R78, R37, and R69 antibodies. As expected, the virus antiserum detected MCDV-S CP1 and CP2, and a third protein slightly larger than CP1 (Fig. 3A). Interestingly, antibodies to all MCDV-S polyprotein regions detected proteins in purified virus preparations. Both the R37 and R69 antibodies detected proteins of approximately 26 kDa (P26) (Figs. 3B and C). The R69 antibodies bound two additional proteins of approximately 30 kDa (P30) and 36 kDa (P36) (Fig. 3C). Similar results were obtained in three independent repetitions of this experiment. The R78 antibodies detected three proteins of approximately 50 kDa (P50), 35 kDa (P35), and 25 kDa (P25) in purified and partially purified virus preparations (Fig. 3D, lanes 2 and 3). This experiment was repeated at least three times with similar results, except that the relative abundance of P50 and P35 varied significantly among virus purifications. The levels of P50 and P35 appeared to vary inversely, with some virion preparations containing high levels of P50 and undetectable levels of P35 (Fig. 3D, lane 4). This suggests that P35 may be derived from processed P50.

To determine whether there was any conservation among MCDV strains for epitopes of the R78 region, the R78 antibodies were used to probe proteins of the MCDV-M1 strain. Interestingly, the R78 antibodies detected a MCDV-M1 protein that migrates slightly slower than the MCDV-S P25 protein (Fig. 3D, lanes 1, 2 and 3). We named this protein P25-M1. Because the R78 antibodies did not react with 50- or 35-kDa proteins in MCDV-M1, the P25 segment appears to be located on a different part of the protein than P50 and P35 and is conserved among MCDV strains.

MCDV-S polyprotein products in plant extracts

To investigate what MCDV proteins accumulate in infected plants, Western blots of protein extracts from infected plant tissues were incubated with MCDV-T, R78, R37, and R69 antibodies cross-absorbed with healthy plant tissue extracts (Fig. 4). The MCDV-T antibodies bound several proteins in MCDV-T- and MCDV-S-infected plant extracts (Fig. 4A, lanes 1 and 2). While there is a clear reaction of the virion antibodies with a protein corresponding in size to CP1 in MCDV-S- and MCDV-T-infected plants, there are several smaller infection-specific proteins present. The origin of these proteins is not clear, although they do appear to be virus related and may be degraded CP. As expected, MCDV-T antibodies did not appear to bind proteins in MCDV-M1-infected plants or healthy plants (Fig. 4A, lanes 3 and 4).

Table 2
Name, sequence, and location of primers used to amplify genome regions corresponding to P78, P37, and P69 of the MCDV-S polyprotein

Protein	Primer	Primer sequence (5′ – 3′)[a]	Location[b]
P78	78_for	gacgataaggatccgATGATGCAGACAAACAACAAC	455 – 475
	78_rev	gccggaggatcc*tca*TTGCTTCACAACTGGTCT	2512 – 2495
P37	37_for	gacgataaggatccgAAGGTGCTTTCGGAACGC	4637 – 4654
	37_rev	gccggaggatcc*tca*CTGGAGCAACTTATAGGT	5596 – 5579
P69	69_for	cgagatctgcagctCAAGTGCTTGATGGAGGTAG	6182 – 6201
	69_rev	gccggatctgcagct*tca*TTGCTTGATGGCTCTT	7993 – 7978

[a] Sequences corresponding to MCDV sequences are indicated in uppercase, added primer sequences are indicated in lower case, stop codons of reverse primers are indicated in italics, and restriction sites used for cloning into pTrcHis B expression vector are underlined.
[b] Location of primers in the MCDV-S nucleotide sequence.

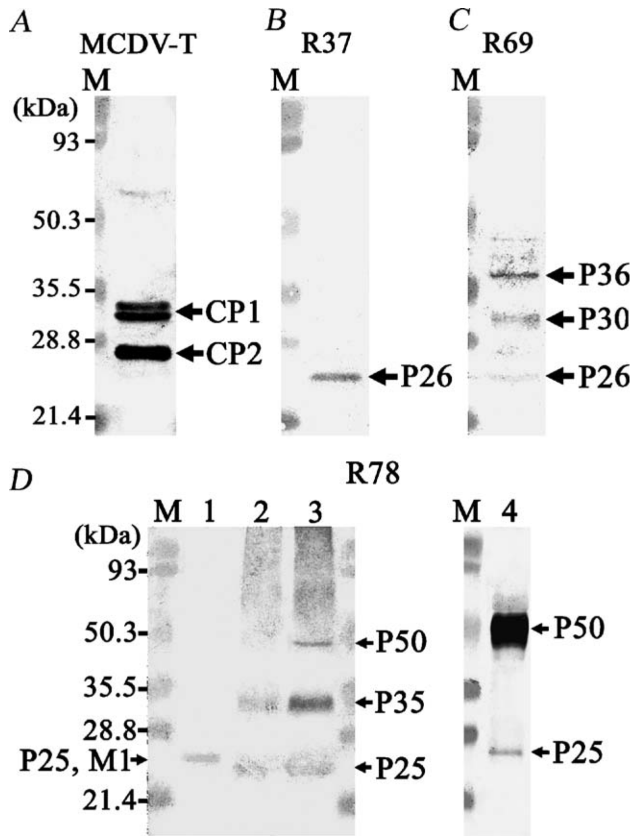

Fig. 3. Detection of MCDV polyprotein products in purified virion preparations on Western blots. Purified MCDV virions (3.75 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with MCDV-T, R37, R69, and R78 antibodies (indicated above the individual panels). For all upper panels, purified MCDV-S was tested. Lanes lower panel: (1) purified MCDV-M1 (1.9 μg); (2) purified MCDV-S (3.75 μg); (3) partially purified MCDV-S (20 μg); and (4) purified MCDV-S (7.5 μg). The molecular weights of marker proteins are indicated (in kDa) to the left of the figure. MCDV proteins are indicated at the right of the panels.

R78 antibodies clearly reacted with a 35-kDa protein, which is likely the same P35 detected in virion preparations (Fig. 3D). The P35 was detected in MCDV-T- and MCDV-S-infected plant extracts, but not in those from MCDV-M1 (Fig. 4B, lanes 1 to 3), confirming the earlier results that MCDV-M1 P35 did not react with R78 antibodies (Fig. 3D, lane 1). R37 antibodies did not bind proteins in protein extracts of MCDV-infected plants (data not shown), suggesting that P26 found in purified virus preparations (Fig. 3B) may not be abundant in MCDV-infected plants or the R37 antibodies are not of good quality. However, the R69 antibodies did bind a 26-kDa protein, probably P26, in MCDV-S- and MCDV-T-infected plant extracts (Fig. 4C, lanes 1 and 2). In addition to P26, the R69 antibodies reacted weakly with a 36-kDa protein (Fig. 4C, lanes 1 and 2) consistent with reactions with purified virus preparations (Fig. 3C). However, P30 present in purified virus preparations was not detected in infected plant extracts by the R69 antibody.

The R78 and R69 antibodies reacted with several higher molecular weight proteins in infected plant extracts. Some of these are also present in healthy plant extracts and hence are likely plant proteins that specifically react with R78 and R69 antibodies. However, some proteins ranging from 50.3 to 93 kDa were detected in infected plant extracts, but not in healthy plant extracts. These proteins might be unprocessed portions of the MCDV polyprotein. In summary, several MCDV proteins including CP1, P36, P35, and P26 were readily detected proteins in infected plants.

Searching for MCDV helper component (HC) protein(s)

Because a virus helper component should be present in the insect vector (Creamer et al., 1993; Hunt et al., 1988), we examined viruliferous leafhoppers for the presence of MCDV-S proteins. Western blots of extracts from leafhoppers fed on healthy or infected maize plants were probed with MCDV-T, R78, R37, and R69 antibodies. No proteins bound MCDV-T, R37, or R69 antibodies (data not shown), whereas R78 antibodies reacted with a protein of approximately 25 kDa, likely P25, in insects that had a 48-h acquisition access period on MCDV-S-infected plants (Fig. 5). P25 was not detected in plant extracts (Fig. 5, lane 2), whereas P35, which was easily detected in MCDV-S-infected plant extracts (Fig. 4B, lane 1, and Fig. 5, lane 2),

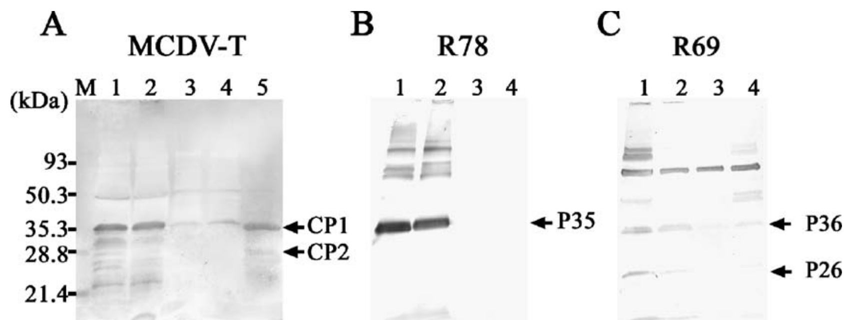

Fig. 4. Identification of MCDV proteins in infected plants. Maize leaf proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with MCDV-T, R78, or R69 antibodies. Blots contain leaf extracts (20-μg protein) from plants infected with MCDV-T (lane 1), MCDV-S (lane 2), and MCDV-M1 (lane 3), healthy control plants (lane 4), and 2.8 μg purified MCDV-S proteins (lane 5). The molecular weights of marker proteins are indicated (in kDa) to the left of the figure, and positions of viral proteins are shown with arrows to the right of the blots.

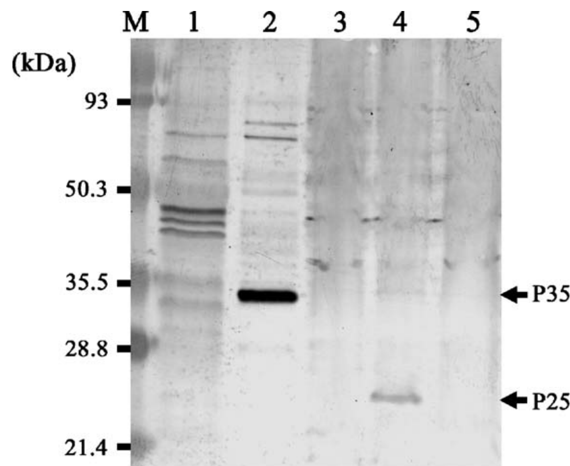

Fig. 5. Identification of R78 antibody-binding proteins in maize and *G. nigrifrons*. Leaf and insect protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with His-R78 antibodies. Lanes: M, marker; 1, leaves from noninfected plants (20-μg protein); 2, MCDV-S-infected leaves (20-μg protein); 3, *G. nigrifrons* fed on noninfected plants (3.75 μg protein); 4, *G. nigrifrons* fed on MCDV-S-infected plants for 48 h (3.75 μg protein); 5, *G. nigrifrons* fed on MCDV-S-infected plants for 48 h and then transferred to healthy plants for 48 h (3.75-μg protein). The size in kDa of marker proteins is indicated at the left of the blot, and the positions of P35 and P25 are indicated with arrows.

was clearly less abundant in viruliferous insects than P25 (Fig. 5, lane 4). These data suggested that P25 accumulated in leafhoppers. However, the leafhoppers lost P25 quickly, as the protein was not detected in leafhoppers that were subsequently transferred to healthy plants for 48 h (Fig. 5, lane 5).

Discussion

The large ORF of MCDV-S encodes a predicted polyprotein of 3457 amino acids. By analogy to other picorna-like plant viruses, one or more virus-encoded proteinases probably cleave this polyprotein into functional structural and nonstructural proteins (Merits et al., 2002; Reichmann et al., 1992). The positions of the CPs were determined experimentally for MCDV-T and MCDV-TN, while a 3C-like protease, an RdRp, and a region with NBS were identified by sequence similarity to other viruses (McMullen et al., 1996; Reddick et al., 1997). The 3C-like protease is likely responsible for cleavage of the coat proteins and RdRp, because 3C-like protease cleavage sites flank the CPs and the RdRp sequences in the ORF. In addition, the N-terminal protein sequences of the three mature CPs correspond to 3C protease cleavage products (McMullen et al., 1996; Reddick et al., 1997).

Although MCDV-T and MCDV-S incite clearly different symptoms in maize and have distinct banding patterns on Western blots, the two viruses have few sequence differences. A third protein, slightly larger than CP1, was detected in MCDV-S virion preparations (Fig. 1), but not in MCDV-T virions. One possibility is that this larger protein is a posttranslationally modified version of CP1. One of the 18 amino acid differences in the MCDV-T and MCDV-S polyproteins results in an amino acid substitution in the CP1 coding region immediately adjacent to a putative glycosylation site. This sequence change could result in greater glycosylation of some MCDV-S CP1 molecules, resulting in the appearance of the larger band. However, because this protein is approximately 35 kDa, an alternative explanation is that the larger protein is P35. Our results show that most of the MCDV-S proteins can be detected in purified virion (Fig. 3), and P35 appears to be abundant in both purified virions and plant extracts. If so, the absence of the P35 protein in purified MCDV-T preparations might be explained by a more readily co-purification of P35 with MCDV-S than with MCDV-T.

Differences in polyprotein amino acid sequences and CP modifications of MCDV-T and MCDV-S may contribute to the differences in symptom severity of these viruses (Shintaku et al., 1992; Sugiyama et al., 2000). However, the most significant differences in sequence between these viruses could be those in the 454-nt 5′ region preceding the polyprotein ORF. In picornaviruses, RNA translation is dependent on an approximately 450-nt segment of the 5′-untranslated region, designated the internal ribosomal entry site (IRES). Introduction of single nucleotide mutations in this region changes symptom severity associated with infection by picornaviruses (Belsham and Sonenberg, 1996; Jackson and Kaminski, 1995; Pilipenko et al., 1999; Rijnbrand et al., 1996). Thus, the three nucleotide differences in the IRES of MCDV-T and MCDV-S may be responsible for the differences in symptom severity of MCDV-T and MCDV-S.

Antibodies to the three protein regions R78, R37, and R69 reacted with proteins in purified virus preparations. MCDV particles are in electron-dense viroplasms that are the sites of genome replication and particle morphogenesis in plant cells (Ammar et al., 1993), and therefore co-purification of P50, P35, P25, P26, P30, and P36 with MCDV virions may not be surprising. The non-CP proteins were not detected on Coomassie- or silver-stained PAGE gels of purified virions. The fact that these proteins could only be detected by immunolabeling suggests that small amounts of these proteins are present in purified virion preparations. Further, the levels of these proteins tended to vary among virion purifications. Co-purification of proteins with virus particles is not an uncommon phenomenon. The ORFIII protein of *Cauliflower mosaic virus* (CaMV) co-purifies with virus particles and is involved in insect transmission (Dautel et al., 1994; Leh et al., 1999). Further, the *Maize dwarf mosaic virus* (MDMV) protein HC-Pro, a multifunctional protein also required for insect transmission of potyviruses (Maia et al., 1996), probably co-purifies with MDMV particles as well, because it reacts with MDMV antibodies, prepared against isolated virus, in purified virus preparations (Redinbaugh et al., unpublished results).

Both R69 and R37 antibodies bound to 26-kDa proteins in virion preparations (Fig. 3). It is possible that a

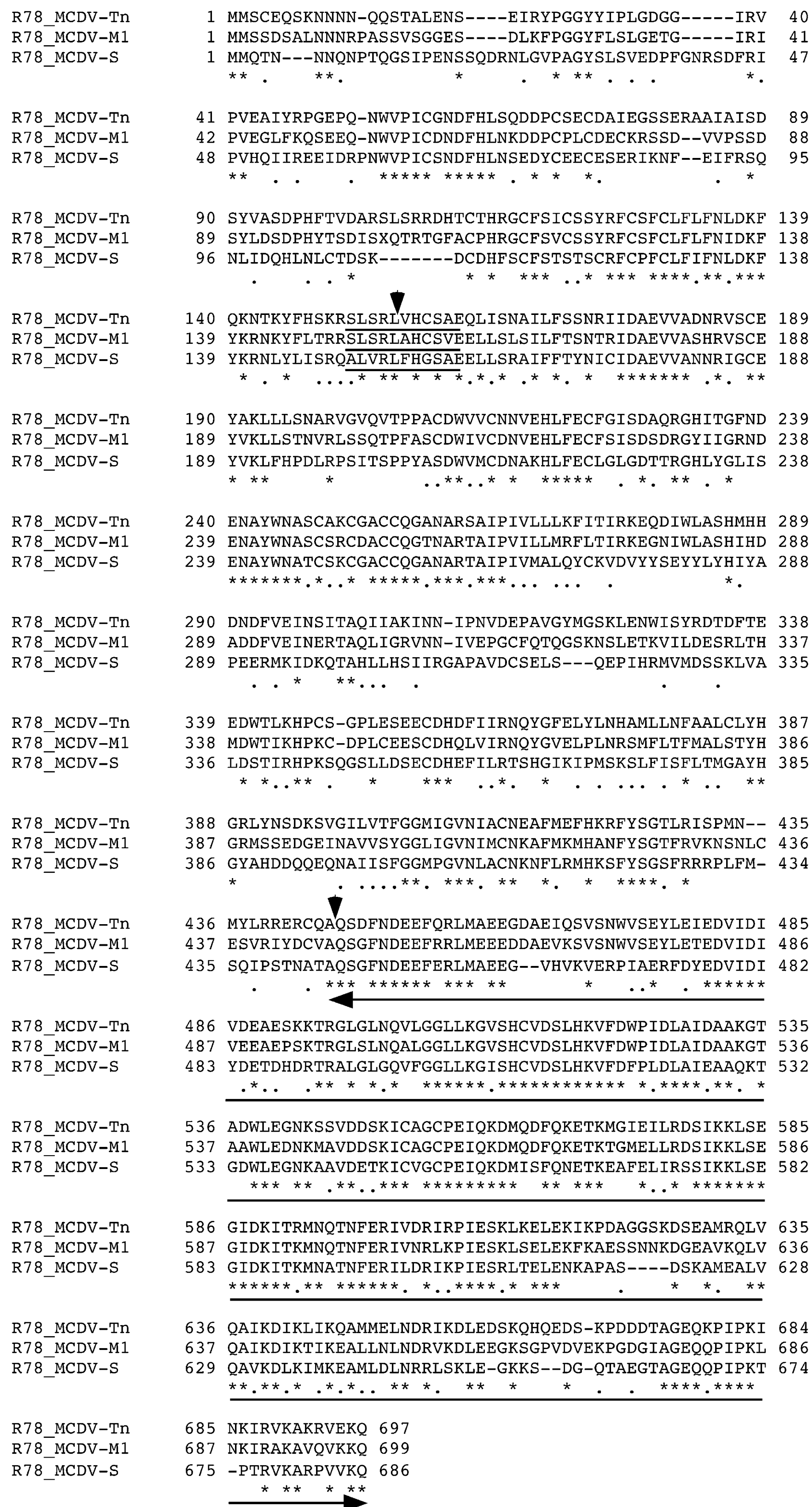

Fig. 6. Alignment of the P78 regions of MCDV polyproteins. The sequences were aligned using Clustal W function of MacVector 3.5. Identical amino acids are indicated with asterisks and similar amino acids with dots. The highly conserved region indicated with a two-headed arrows is located adjacent to CP2 (Fig. 2) and possibly corresponds with the P25 of MCDV-S (243 amino acids) and P25-M1 of MCDV-M1 (254 amino acids). The putative autocatalytic cleavage sequences are underlined. The cysteine protease site that may cleave R78 into P25 and P50, and the autocatalytic cleavage site that may produce P35 from P50 are indicated with arrowheads.

single P26 protein spanning the 7.2-kDa NBS-like region (Fig. 1) reacts with both antibodies. The R69 antibodies also detected two other proteins, P30 and P36 for a cumulative size of approximately 66 kDa. It is possible that P30 and P36 are adjacent to each other in R69 next to the C3-like protease, with the N-terminal portion of R69 reacting with a 26-kDa NBS-containing protein. Because R37 antibodies react with the same 26-kDa protein, a region of approximately 300 amino acids (approximately 33 kDa) between CP1 and P26 remains undetected in virions, plants, and insects. An alternative possibility is that the antisera react with two different 26-kDa proteins. In this case, the 26-kDa protein detected with the R69 antisera is likely to be a proteolytic product of either P30 or P36, and the 26-kDa protein detected with R37 antisera could be located anywhere within the R37 and NBS regions. Further research is required to distinguish between these two possibilities.

Antibodies to the R78 region detected P50, P35, and P25. A putative cysteine protease cleavage site (Q/S) is present after amino acid 444 of the MCDV-S sequence, and this site is conserved among MCDV-S, MCDV-M1, and MCDV-TN (Fig. 6) (McMullen et al., 1996; Reddick et al., 1997). Cleavage at this site and the previously determined conserved site at the N-terminus of CP2 results in proteins of 51 and 27 kDa for MCDV-S and MCDV-TN. The corresponding MCDV-M1 region is 11 amino acids longer and has a predicted molecular weight of approximately 28 kDa, consistent with the slightly slower migration of P25-M1 from MCDV-M1 relative to MCDV-S (Fig. 3). NetPicoRNA protease prediction software (Blom et al., 1996) predicts an autocatalytic cleavage site at "ALVRLFHGSAE" after amino acid 154 of the MCDV-S polyprotein that is conserved among the MCDV proteins (Fig. 6). Cleavage at this site would result in production of P35 and an 18-kDa protein. This predicted cleavage is consistent with the observed inverse variation in P50 and P35 among experiments. However, the 18-kDa protein was not observed in our experiments. This protein may be unstable or it may lack antigenicity.

The putative P25 regions are significantly more conserved among MCDV isolates than the adjacent N-terminal portions (Fig. 6), consistent with our results showing that P25, but not P50 or P35, has epitopes that are conserved among the MCDV isolates. The putative MCDV-S P25 region is 76% identical to the corresponding region of MCDV-TN and 71% to that of MCDV-M1, whereas the putative MCDV-S P50 region is 56% similar to that of MCDV-TN and 58% to that of MCDV-M1. Also, consistent with P25 being adjacent to the CP2 is the slightly decreased mobility of P25-M1 from MCDV-M1. Thus, P25 is apparently located on a different part of the polyprotein than P50, and P35 may be an autocatalytic cleavage product of P50. Indeed, the cumulative predicted molecular weights of P50 and P25 (77 kDa) are similar to the calculated molecular weight of R78.

MCDV requires a helper component (HC) for transmission by insects (Creamer et al., 1993; Hunt et al., 1988). Because P25 of MCDV-S accumulates in *G. nigrifrons* fed on infected plants, but not in insects fed on healthy plants, we hypothesize that P25 might be a helper component of MCDV. Further support of a helper component role for P25 comes from the fact that P25 cannot be detected in leafhoppers at 48 h after acquisition (Fig. 5), corresponding with the lack of continued MCDV transmission by *G. nigrifrons* by about 48 h after virus acquisition (Creamer et al., 1993). Whereas MCDV-S and MCDV-M1 are weakly related serologically, the viruses complement one another's HC for transmission by *G. nigrifrons* (Creamer et al., 1993), consistent with the relatively higher conservation of the virus polyprotein in the region putatively corresponding to P25. In addition, *G. nigrifrons* transmits MCDV-S, MCDV-T, MCDV-TN, and MCDV-M1 (Gingery and Nault, 1990; Nault et al., 1973; Reddick et al., 1997), suggesting some degree of conservation of transmission determinants among the virus isolates. Further research is needed to confirm and elucidate the mechanism of action for the MCDV HC.

Material and methods

Virus source and propagation

Isolates of MCDV-T and MCDV-M1 were obtained from Johnson grass rhizomes collected near Portsmouth, OH, in 1972 (Nault et al., 1973) and 1988 (Gingery and Nault, 1990), respectively. A more severe isolate, MCDV-S (or MCDV-White Stripe, MCDV-WS), was obtained from an infected field corn. All isolates were maintained in the inbred maize (*Zea mays* L., Oh28) by serial leafhopper transmission. *G. nigrifrons* was reared in cages containing oats (*Avena sativa* L.) and the sweet corn inbred Oh28. Adults used for acquiring MCDV from symptomatic plants were 1–3 weeks old. Virus-infected source plants used in all experiments were Oh28 maize.

Virus purification

Purified MCDV virion preparations were obtained using the procedure of Gingery and Nault (1990) with minor modifications. At 4–8 weeks after inoculation, leaves (approximately 90 g) from symptomatic plants were homogenized with 4 ml/g FW 0.1 M potassium phosphate, pH 7.0 containing 0.5% β-mercaptoethanol. After purification, virion-containing pellets recovered from gradient fractions were resuspended in 0.3 M potassium phosphate, pH 7.0. Partially purified virus preparations were obtained by resuspending the pellet from the first high-speed ($30000 \times g$) centrifugation in the same buffer. The amount of protein was determined with the dye binding method (Redinbaugh and Campbell, 1985).

Genome sequencing and map construction

RNA was extracted from partially purified MCDV-S virions. The green pellet was resuspended in 25 ml of guanidine thiocyanate extraction buffer by gentle agitation overnight at 4 °C, homogenized using a tissue homogenizer for 45 s, layered onto 9 ml 5.7 M cesium chloride pad, and centrifuged at 25 000 × *g* for 24 h at 4 °C. The clear pellet was resuspended in 500 μl of deionized H_2O, extracted with phenol-chloroform extraction, and precipitated with ethanol/Na-acetate (Sambrook et al., 1989) before resuspension in deionized water.

Nucleic acid sequences were determined by primer walking using a Perkin-Elmer ABI377 Prism DNA sequencer and ABI BigDye Terminator Reaction kit (Applied Biosystems, Foster City, CA). The sequences were initially edited and analyzed using Sequencer (version 4.1) software (Gene Codes Corp., Ann Arbor, MI). Further analysis and sequence comparisons were performed using MacPhred-MacPhrap (Ewing and Green, 1998; Ewing et al., 1998) and MacVector (Accelrys, San Diego, CA) software and BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) searches against the National Center for Biotechnology Information (NCBI) GenBank nonredundant (nr) database.

The MCDV-S genome sequence was determined from overlapping RT-PCR products synthesized with the ThermoScript RT-PCR system (Invitrogen Corp., Carlsbad, CA) either directly or after cloning into pGEM-T (Promega Corp., Madison, WI). For each PCR product, sequences of both strands for two independent clones were determined. The 5′ end of the MCDV-S genome was cloned using 5′ RACE (Invitrogen), and the 3′ end sequence was obtained from an RT-PCR product amplified with a NNT_{15} degenerate primer.

Protein expression and antiserum production

The three undefined regions of the MCDV-S genome were amplified using primers listed in Table 2, and PCR products were cloned into the *E. coli* expression vector pTrcHis B (Invitrogen) after which all three inserts were sequenced for verification. The pTrcHis B constructs were expressed in *E. coli* XL1-Blue (Stratagene Corp., La Jolla, CA). After 3-h growth and 3-h induction with isopropyl-β-D-thiogalactoside (IPTG, 0.1 mM), bacterial suspensions were harvested, sonicated to macerate the bacteria, and purified by affinity binding using Ni-NTA resins (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. Before antibody production, purity of the three His-fusions was confirmed on Coomassie brilliant blue-stained gels and Western blots probed with anti-His IgG (data not shown). For antisera production, New Zealand rabbits were immunized with five monthly injections of the purified fusion protein using a standard protocol (Harlow and Lane, 1988).

Protein electrophoresis

Protein extracts were prepared by grinding virus preparations, fresh maize leaves, and insects in 2 ml 25 mM Tris, pH 8, 0.75 M NaCl/g FW tissue. Proteins were mixed with equal volumes of protein dissociation buffer (0.5 M Tris–HCl, pH 6.8; 4% SDS; 10% β-mercaptoethanol; and 10% glycerol), incubated at 100 °C for 10 min, and separated on 12% vertical denaturing polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Proteins were stained with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose membranes (0.2 μm, Bio-Rad) according to the manufacturer's recommendations. Membranes were blocked with 5% nonfat dry milk in PBS buffer for 1 h, washed in PBS containing 0.01% Tween 20 (PBS-T) three times for 5 min, incubated with 1:500 dilution of primary antibody in SEB buffer (PBS-T containing 2% polyvinyl pyrrolidone and 0.2% ovalbumin) for 2 h, washed in PBS-T three times for 5 min each, and finally incubated with 1:2000 dilution alkaline phosphatase-conjugated goat anti-rabbit IgG in SEB buffer for 2 h. Membranes were rinsed in PBS-T buffer four times before enzymatic development with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Bio-Rad). Western blots of plant extracts were probed with IgG purified (McDaniel and Gordon, 1989) from antisera that were previously cross-absorbed with extracts from healthy plants. The probes contained 3–8 μg purified IgG/ml SEB.

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