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Short communication

A carlavirus-specific PCR primer and partial nucleotide sequence provides further evidence for the recognition of cowpea mild mottle virus as a whitefly-transmitted carlavirus

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Abstract

Cowpea mild mottle virus (CMMV) has physicochemical properties typical of carlaviruses, but has remained unclassified due to a number of unusual properties, including no serological cross-reaction with 18 carlaviruses; production of brush-like inclusion bodies *in vivo*; and the ability to be transmitted by whiteflies (*Bermisia tabaci*). In this paper we report the use of a carlavirus specific PCR primer to identify CMMV as a member of the carlavirus group. This is confirmed by nucleotide sequence (958 nucleotides) from the 3′ terminal region of CMMV RNA which contains a partial open reading frame (ORF) having high similarity with the coat proteins of other carlaviruses. The sequence also contains an 11.7K ORF at the 3′ terminus, containing a 'zinc-finger' motif which is unique to carlaviruses.

Cowpea mild mottle virus (CMMV) has physicochemical properties which resemble those of members of the carlavirus group, namely the presence of filamentous particles *c.* 650 nm in length, consisting of a coat protein of 31-33K and a single-stranded RNA of Mr 2.5 × 10^6 [Jeyanandarajah and Brunt, 1993]. However, unlike other members of the carlavirus group which are transmitted non-persistently by aphids [Foster, 1992], CMMV is unusual in that it is transmitted in a non-persistent manner by whiteflies (*Bemisia tabaci*) [Jeyanandarajah and Brunt 1993]. In addition, CMMV has several other unusual properties compared with other carlaviruses, which include, no serological relationships with 18 recognised members of the carlavirus group, and the induction of unusual brush-like intracellular inclusions in infected plants [Brunt *et al.*, 1983]. It has been suggested that CMMV remain unclassified until the taxonomic significance of these differences have been investigated further [Jeyanandarajah and Brunt, 1993].

We report here attempts to develop a rapid and specific identification test for carlaviruses which can also be used to determine whether CMMV and similar unclassified viruses are species of the carlavirus genus. The test is based on the polymerase chain reaction (PCR) using primers to conserved and unique sequences in carlavirus RNAs.

The overall genome organisation of carlaviruses is very similar to that of potexviruses with the virus genes of both genera being arranged (from 5′ to 3′) as replicase, a triple gene block (25K, 12K, and 7K), and coat protein open reading frame, with extensive similarity at the amino acid level between the equivalent proteins of potex- and carlaviruses. However, in addition to these proteins, carlavirus genomes contain an additional protein encoded by an open reading frame

(ORF) at the 3′ terminus downstream from the coat protein gene. This ORF potentially encodes a protein of approximately 11K in size and contains domains which are highly conserved between all carlaviruses with four cysteine residues in a pattern of C–X_2–C–X_{12}–C–X_4–C. These cysteine residues are arranged as a zinc-finger, which conforms to a consensus sequence found in many nucleic acid binding proteins [Foster, 1992]. The location of this ORF between the coat protein gene and the poly(A) tail differentiates carlaviruses from potexviruses and other plant RNA viruses.

Sequences within this gene therefore seemed ideal for the design of a PCR primer which may be used both to detect carlaviruses and to identify unknown viruses as members of the carlavirus group. When sequences representing the region from the initiation codon of the 11K gene to the poly(A) tail from a number of published sequences of known carlaviruses were aligned an area of high sequence similarity was evident at the 3′ region of the 11K gene. In 6 of 8 published carlavirus sequences, a highly conserved sequence of GGAGTAACC (or T) GAGGTGATACC was evident close to the termination codon for the 11K gene, *c.* 120bp from the 3′ terminus (see Fig. 1A). In some carlaviruses, the termination codon for the 11K was contained within this sequence, in others the coding region continued through this region with the termination codon being located further downstream. It is unclear at this stage why such high similarity is evident at the nucleotide level between these carlaviruses, within a region which may either be coding or non-coding. However, this seemingly unique and highly conserved block of 20 nucleotides, provides an ideal opportunity to test whether a PCR-based experiment could be designed for identifying an unknown virus as a member of the carlavirus group.

To this end a primer (designated Carla-Uni) was made (Fig. 1) which represented the sequence described above.

We describe here an assessment of this primer when tested with definite carlaviruses, other viruses so classified only on the basis of their physicochemical and/or serological properties, possible carlaviruses such as CMMV and viruses of the potex- and potyvirus genera. The amplification of CMMV RNA by the primer provided evidence that CMMV is a carlavirus, a conclusion supported by the similarity of its 3′ nucleotide sequence to those of definite carlaviruses. It was decided to evaluate the primer Carla-Uni RT-PCR test using RNA extracted directly from infected tissues as in the process of identifying an unknown virus, the starting material is often infected plant tissue with no known purification method for the virus.

Approximately 1 cm^3 of infected leaf tissue was ground to a fine powder using a wooden applicator stick, in a 1.5 ml Eppendorf tube cooled in liquid nitrogen. Ground leaf tissue was extracted with 300 μl of extraction buffer (2% SDS, 0.1M Tris-HCI pH 8, 2 mM EDTA), 150 μl of phenol pH4.5 and 150 μl of chloroform. The preparation was mixed well and incubated at 70 °C for 56 min, before centrifugation. The supernatant was transferred to a clean microfuge tube and 2 μl of 1M DTT and 1 μl of RNasin (40U/μl) were added. Reverse transcription and PCR inhibitors were removed by purification of polyadenylated RNAs from the supernatant using Oligo (dT) 25 Dynabeads (Dynal A/S, Oslo, Norway) as recommended by the manufacturers. Eluted RNA (20 μl) was stored at −80°C before use as template for reverse transcription.

Reverse transcription reactions were carried out in volumes of 25 μl containing 1 × AMV buffer (Promega), 1 mM of each dNTP, 15U AMV reverse transcriptase (Promega), 10U RNasin (Promega) and 4 μM of each primer, and template RNA (10 μl of purified extract). For carlavirus and potyvirus amplifications the cDNA first strand oligo d(T) anchored primers of Pappu *et al.* [1993] were used: these were designated CN47, CN54 and CN55 (these primers contain 5′T_{21} and either A, C or G at the 3′ end), and for PVX, the primer used was designated PVX-3′. Template RNAs were incubated at 85 °C for 5 min, then transferred to a thermal cycler set at 42 °C, reverse transcriptase reagents added, and the mixture incubated for 30 min.

PCR reaction volumes (25 μl) contained 1 × PCR buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6U Taq polymerase (Promega), 2 μl of the 25 μl of cDNA produced in the RT reaction described above and appropriate combinations of PCR primers.

PCR amplifications were performed using Techne PHC 3 thermal cyclers, under the following conditions; denaturation: 94 °C, 30 sec; annealing: 42 °C (potyvirus primer), 50 °C (Carla-Uni primer), 55 °C (PVX primers), 1 min; extension: 72 °C, 1 min (PVX and Carla-Uni), 2 min (potyvirus); for 35 cycles. Amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5 × TBE and visualised at 302nm after staining in ethidium bromide (0.5 μg/ml).

As positive controls RT-PCR reactions were carried out on three carlaviruses which contained the sequence

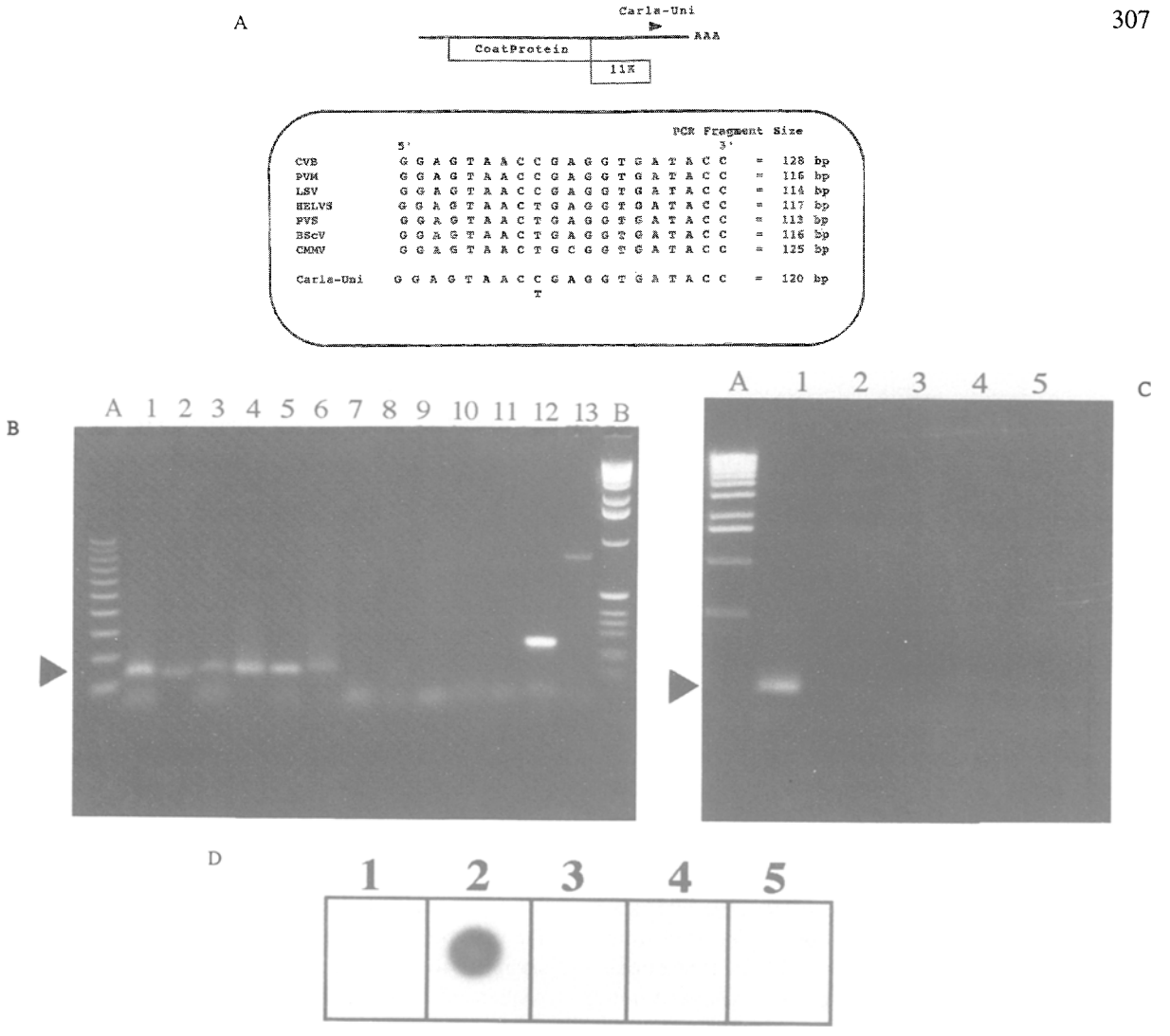

Fig. 1. Position of the sequence of the primer Carla-Uni on the carlavirus genome. The degree of similarity between seven carlaviruses for which sequence information has been reported is shown within the enclosed box. The sequence and distance from the start of the poly(A) tail is shown for chrysanthemum virus B [CVB; Levay and Zavriev, 1991], potato virus M [PVM; Zavriev *et al.*, 1991], lily symptomless virus [LSV; Memelink *et al.*, 1990], Helenium virus S [HeLVS; Foster *et al.*, 1990], potato virus S [PVS; Foster and Mills, 1992; MacKenzie *et al.*, 1989] [sequence in this region is identical in both ordinary and Andean strains of PVS], blueberry scorch virus [BScV; Cavileer *et al.*, 1994] [sequence is present in 3 strains of the virus] and cowpea mild mottle virus (CMMV) (from information reported in this paper); (b) Agarose gel analysis of PCR products generated using Carla-Uni and anchored oligo-d(T) primers on first-strand cDNA generated from RNA isolated from plant material infected with a range of viruses (known carlaviruses, possible carlaviruses and viruses of other genera with filamentous particles), as outlined below. Lane A, molecular weight markers (Superladder; Advanced Biotechnologies); lane B, molecular weight markers (kilobase ladder; Gibco BRL). Lanes 1–12 were PCR reactions performed with Carla-Uni and anchored oligo-d(T) primers CN47, CN54 and CN55. Closed arrow indicates the predicted PCR product of approximately 120bp which should be obtained with Carla-Uni and oligo-d(T). Lane 1, potato virus S; lane 2, potato virus M; lane 3, lily symptomless virus; lane 4, American hop latent virus; lane 5, hop mosaic virus; lane 6, cowpea mild mottle virus; lane 7, narcissus latent virus; lane 8, Maclura mosaic virus; lane 9, potato virus X; lane 10, potato virus Y; lane 11, negative control (water). Lane 12, potato virus X positive control PCR with PVX specific primers. Lane 13, potato virus Y positive control PCR with CN-48 potyvirus primer and CN-47, CN-54 and CN-55; (C) Agarose gel analysis of PCR products and control PCR reactions generated from first strand cDNA primed with oligo-d(T) generated from RNA extracted from purified particles of CMMV. Lane A – molecular weight markers (kilobase ladder; Gibco BRL). Lane 1, PCR reaction containing cDNA and both primers Carla-Uni and oligo-d(T)-Not; lane 2, PCR reaction containing cDNA and oligo-d(T)-Not alone; lane 3, PCR reaction containing cDNA and Carla-Uni alone; lane 4, PCR reaction containing both primers both no cDNA; lane 5, PCR reaction containing cDNA but no primers. Closed arrow indicates the PCR product of approximately 120bp which should be obtained with Carla-Uni and oligo-d(T)-Not; (D) RNA dot-blot analysis using radiolabelled 120bp PCR fragment generated from CMMV as probe. 1 μg of each RNA in a volume of 5 μl was dotted onto Hybond-N (Amersham) previously pre-soaked in 2 × SSC and air dried, UV crosslinked and hybridised with the CMMV 120bp fragment radiolabelled with ^{32}P, as previously described by Foster and Mills [1990]. The blot was washed, dried and exposed overnight to X-ray film. Sample 1, Maclura mosaic virus RNA; sample 2, cowpea mild mottle virus RNA; sample 3, narcissus latent virus RNA; sample 4, brome mosaic virus RNA (BMV); sample 5, tobacco mosaic virus RNA (TMV).

represented in the primer Carla-Uni, namely potato virus S (PVS), potato virus M (PVM) and lily symptomless virus (LSV). As can be seen from lanes 1, 2 and 3 in Figure 1B, all three generated bands of the predicted sizes. Reactions were also carried out with American hop latent virus (AHLV) and hop mosaic virus (HMV), two viruses which have been assigned to the carlavirus group on the basis of typical biological and physicochemical properties [Adams and Barbara, 1980, 1982]. In these reactions bands were also generated of the correct size (Fig. 1B, lanes 4 & 5).

When RT-PCR was carried out on CMMV a correctly-sized 120bp band was obtained (Fig. 1B, lane 6). However, for narcissus latent virus (NLV) and Maclura mosaic virus (MacMV), two viruses which in the past have been tentatively assigned to both the carla- and potyvirus groups, but remain unclassified [Shukla *et al.*, 1994], no bands of strong intensity and the correct size were generated (FIg. 1B, lanes 7 & 8).

To test the specificity of Carla-Uni further, RT-PCR reactions were carried out on plant material infected with two filamentous viruses, potato virus X (PVX) and potato virus Y (PVY) the type members of the potexvirus and potyvirus genera respectively, and no bands were generated (Fig. 1B, lanes 7 & 10). This is also true when PCR reactions were carried out on a water control (no cDNA, Fig. 1B, lane 11). To confirm that cDNA had been generated from the PVX and PVY infected material, RT-PCR reactions were carried out using control primers designed to a region upstream from PVX coat protein ORF (PVX-5′ & PVX-3′, sequence of primers not presented), and in the case of PVY, oligo-d(T) and a potyvirus universal primer located within the coat protein (CN48, 5′-TGGTG1AT2GA3AATGG-3′, code 1 – C or T, code 2 – A or C or T, code 3 – A, C, G, or T, as described by Pappu *et al.*, 1993). In both cases bands of the correct sizes were obtained (Fig. 1B, lanes 12 & 13).

To verify that the DNA product generated by RT-PCR from CMMV-infected leaf material was of viral origin, similar experiments were carried out on RNA extracted from purified CMMV particles. As can be seen in Figure 1C, a PCR product of *c.* 120 bp was produced in PCR reactions containing first-strand cDNA to viral RNA primed with oligo-d(T), and PCR primers oligo d(T)-Not (5′-CAATTCGCGGCCGCT $_{18}$–3′) and Carla-Uni. No bands were evident in any control PCR reactions. Further evidence that this band was generated from CMMV sequences was provided from northern dot blots. When the band was excised from agarose gels, radio-labelled by random primed incorporation using ^{32}P-CTP and used to probe a range of viral RNA dotted onto nylon membranes, the only signal obtained after hybridisation was from RNA extracted from CMMV, with no signals from RNA extracted from tobacco mosaic tobamovirus (TMV), brome mosaic bromovirus (BMV), NLV or MacMV (see Fig. 1D). Thus the PCR product generated using these primers apparently provides a probe for detection and indicates that the DNA product generated by RT-PCR was of viral origin.

As this is the first report of the use of this carlavirus-specific PCR primer to assign a virus to the carlavirus genus, dsDNA clones representing the 3′ terminal region of CMMV were generated by conventional methods and sequenced to determine if the Carla-Uni sequence and an ORF representing the 11K, which is unique to the carlavirus group, were present.

RNA extracted from purified CMMV was used as a template for the generation of ds-cDNA using standard techniques with an oligo-d(T) primer (Promega cDNA synthesis kit) and after the addition of *BstX* I linkers, cloned into *BsTX* I cut pcDNAII (Invitrogen). Colonies were screened with radiolabelled first-strand cDNA to CMMV RNA, with positives being sized and sequenced. The largest insert within this first set of clones was *c.* 500bp which covered the 11K ORF and 3′ untranslated region. To extend the sequence into the coat protein region a complementary primer was designed to the 5′ terminal region of the initial clones sequenced, used to prime cDNA to CMMV RNA; the ds-cDNA generated was cloned into *Sma* I cut pUC18 (Pharmacia). This second set of clones extended the viral region sequenced to *c* 958bp of the 3′ terminal region of CMMV as shown in Figure 2A. Contained within this region, was a partial ORF of 19.5K and a 3′ terminal ORF of 11.7K, which displayed high similarity with the C-terminal region of the coat protein and the 11K ORFs of two previously sequenced carlaviruses. A comparison of the amino acid sequences of the CMMV ORFs and two carlaviruses, PVS [Foster and Mills, 1992; MacKenzie *et al.*, 1989] and HeLVS [Foster, *et al.*, 1990], showed that CMMV has 56% similarity with PVS and 54% with HeLVS within the coat protein at the amino acid level, and 48% with PVS and 46% with HeLVS over the 11K ORF (Figs. 2B, 2C). The gene arrangement, amino acid similarity and presence of the carlavirus-specific 11K ORF, clearly provides strong evidence that CMMV is a carlavirus. This confirms the classification suggested by the PCR results using the Carla-Uni primer, which can be

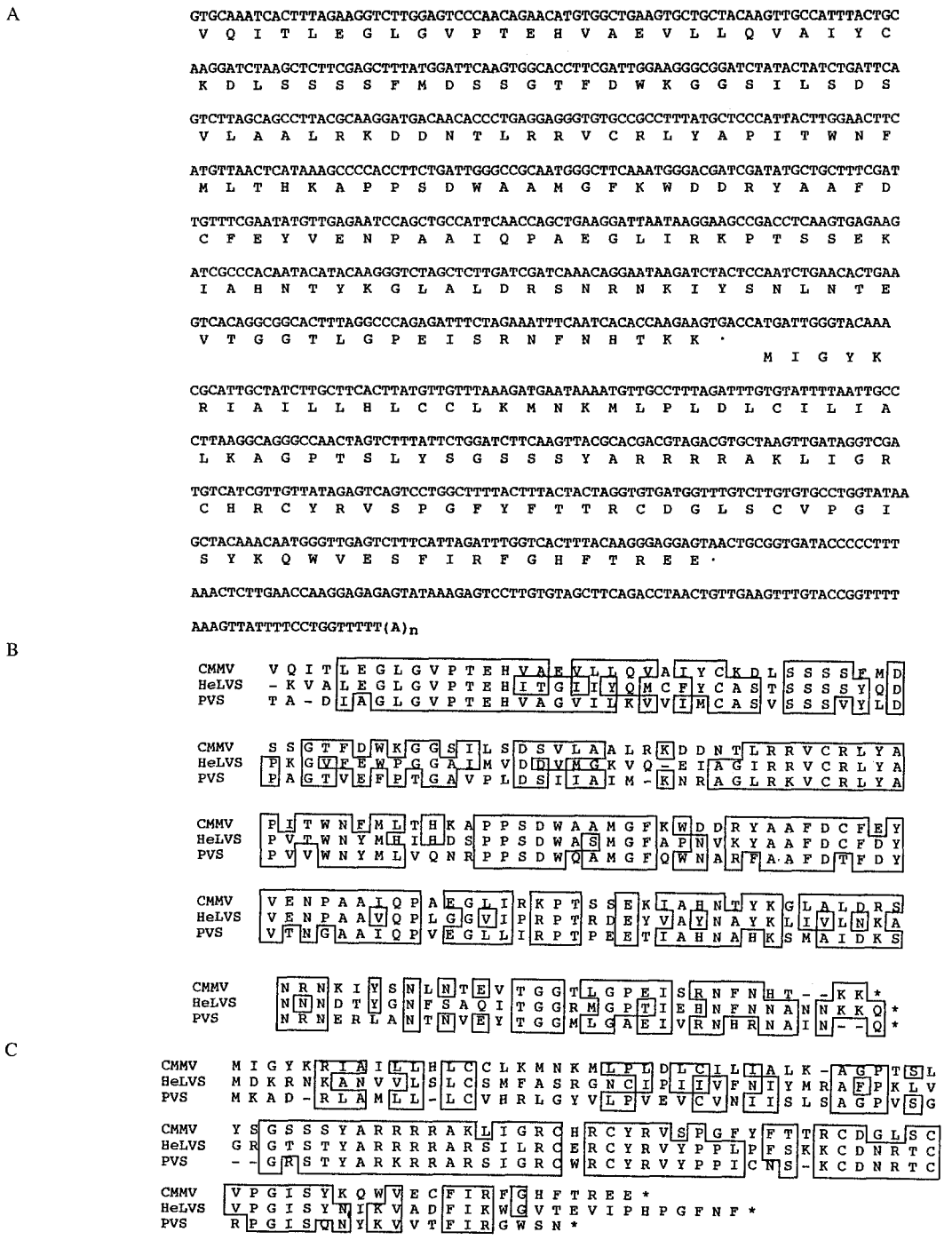

Fig. 2. Nucleotide sequence of CMMV 3′ terminal region and amino acid similarities with other carlaviruses. (A) The DNA sequence corresponding to the 3′-terminal region of CMMV positive-sense RNA, with the derived amino acid sequence indicated below. The sequenced was obtained from at last two independent clones, sequenced on both strands. A partial ORF of approx. 19.5K is evident which has similarity with other carlavirus coat proteins (see Figure 2B) followed by an ORF encoding a cysteine-rich protein of 11.7K; (B) Alignment of the partial ORF of CMMV with the C-terminal regions of the coat protein genes of HeLVS and PVS> Gaps (–) have been introduced for maximum alignment, with identical amino acids boxed, and termination codons indicated by asterisks(*); (C) Alignment of the predicted amino acid sequences encoded by the 3′ 11K ORF of CMMV with the equivalent ORFs of HeLVS and PVS. Gaps (–) have been introduced for maximum alignment, with identical amino acids boxed, and termination codons indicated by asterisks(*).

located within the CMMV sequence (see Fig. 2A), and also aligned with other carlavirus sequences in Fig. 1A.

In this paper we have described the design and testing of a PCR primer whose sequence is common to a large number of carlaviruses. While this primer sequence is not present on the genomes of carnation latent virus [CLV; Meehan and Mills, 1991] and poplar mosaic virus [PMV; Henderson *et al.*, 1992], it is present on 82% of all carlaviruses sequenced or tested to date. We therefore consider it a useful tool in assigning unknown viruses to the carlavirus group, although negative results would clearly require further investigation.

To conclude our results using this primer produced positive results for 3 carlaviruses for which the sequence was already known, as well as producing further evidence that AHLV and HMV, which to date had been assigned to the carlavirus group on the basis of physicochemical and serological properties, are correctly classified. When the Carla-Uni primer was used on three viruses of uncertain taxonomy, a positive result was obtained for CMMV and negative results for NLV and MacMV. To further investigate the results obtained for the latter three viruses and to help validate the use of the PCR primer Carla-Uni, the genomes of all 3 have been cloned from conventional cDNA and sequenced. The sequences of NLV and MacMV will be presented elsewhere, although it is relevant to mention here that neither of these viruses are in fact carlaviruses. The nucleotide sequence of the 3′ terminal region of CMMV genomic RNA reported in this paper, clearly indicates that CMMV is indeed a whitefly-transmitted carlavirus.

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