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Development and use of detection methods specific for *Cucumber vein yellowing virus* (CVYV)

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Abstract

Two methods for the detection of *Cucumber vein yellowing virus* (CVYV) on infected plants were developed, based on the information provided by cDNA clones covering the 3′-end of the genome of a Spanish isolate (CVYV-AILM). The sequenced portion of the CVYV-AILM genome showed a 96.6% aminoacid identity with that of a reported sequence of another CVYV isolate from Israel (Lecoq et al., 2000). The first detection method used a RNA specific probe for hybridization with nucleic acids extracted from infected plants. The probe was complementary to a portion of the CVYV genome including the C-terminal part of the NIb and most of the coat protein (CP) coding regions. The second detection method employed polyclonal antisera raised against recombinant viral CP expressed in bacteria. The specific antibodies were used to detect the presence of virus particles in plant extracts. Both procedures resulted in a highly specific detection of CVYV in plants infected with different isolates of the virus. No interference was observed with other cucurbit-infecting viruses. Sensitivities achieved were sufficient for routine diagnosis of the presence of the virus in plants.

Introduction

Cucumber vein yellowing virus (CVYV) causes a whitefly-transmitted disease in cucurbits, being responsible for important economical losses in several crops. The virus has been included taxonomically in the genus *Ipomovirus*, family *Potyviridae* (Lecoq et al., 2000). This designation is supported by several lines of evidence, including biological properties, cytological observations and molecular data. First, CVYV is vectored by *Bemisia tabaci* whiteflies in a semipersistent manner (Mansour and Musa, 1993), a feature shared with other members of the genus (Jones, 2003). Second, under electron microscopy the presence in infected plant cells of typical pinwheel structures was observed (Lecoq et al., 2000; unpublished observations). The occurrence of pinwheels is unique and characteristic of all members of the family and thus it is considered a clear taxonomical mark for allocation into the *Potyviridae* (Shukla et al., 1994). Finally, molecular data derived from comparison of a partial sequence of the 3′ region of the viral genome of the Israelian isolate CVYV-Isr have confirmed the closest relationship of CVYV with ipomoviruses (Lecoq et al., 2000).

Although initially restricted to the Middle East, an epidemic of CVYV was reported in greenhouse cucumber crops in Almería (Southeast Spain), a

major productive region for protected horticultural crops (Cuadrado et al., 2001a). From this initial outbreak, CVYV has extended to other crops such as melon and watermelon in this and other Spanish regions, where it is threatening cucurbit production.

One initial limitation for any control strategy of CVYV is the scarce availability of rapid, reliable and inexpensive specific detection methods. Once CVYV taxonomy was clarified and a partial nucleotide sequence was accessible, molecular methods of detection become feasible, for instance RT-PCR techniques (Cuadrado et al., 2001b). The importance of a sensitive diagnostic tool is illustrated by the discovery of the ability of CVYV to infect non-cucurbit weed plants (Janssen et al., 2002), a fact that needs to be carefully considered in epidemiological studies. However, the current diagnosis methods based on RT-PCR might be difficult to implement in large scale control programmes, and new and simpler detection tools might serve to facilitate its widespread use by all agents involved in controlling the disease. Particularly, serological tools for detection of the virus had not been developed. This seemed to be due to the reported difficulties in purifying CVYV virions (Sela et al., 1980; Lecoq et al., 2000) that had precluded the preparation of good antisera by direct immunization of animals with virus particles. To overcome these limitations, we decided to explore alternatives to obtain good diagnostic systems for this virus. The interest in new methods for the rapid detection of CVYV was shown in a recent publication that describes the use of hybridization with cDNA probes (Rubio et al., 2003).

In this work we report the development and use of two new detection tools for CVYV. The first is based in hybridization with a CVYV specific RNA probe, which was used successfully to detect the presence of the virus in purified nucleic acids or tissue prints obtained from field infected plants. The second detection tool developed uses polyclonal antisera raised against the structural protein of the virus particles, the coat protein (CP), which was expressed in bacteria as a recombinant fusion product. Different methods based on these new tools resulted in highly specific detection of CVYV in plants infected with different isolates, and no interference was observed with other commonly found cucurbit-infecting viruses.

Materials and methods

Plant, virus and insect cultures

The CVYV-AILM isolate was obtained from cucumber plants grown in EI Ejido (Almeria, Spain) and was maintained by mechanical inoculation or whitefly transmission in *Cucurbita pepo* cv. Diamant (Semillas Fitó, Spain) or *Cucumis sativus* cv. SMR-58. Other CVYV isolates from Almeria and from Israel (CVYV-Isr) were made available by Dr. Herve Lecoq (INRA-Avignon, Montfavet, France) and maintained in *Cucumis melo* cv. Vedrantais. The crinivirus *Cucurbit yellow stunting disorder virus*-AILM (CYSDV-AILM; Aguilar et al., 2003) was maintained in *C. melo* cv. Amarillo (C-250, La Mayora germplasm collection, Malaga, Spain) through transmission by *Bemisia tabaci*. Isolates of other cucurbit-infecting viruses were provided by Dr. Enrique Moriones (EELM, CSIC, Malaga, Spain). These include three potyviruses and one cucumovirus: the Italian isolate Z43 of *Papaya ringspot virus* PRSV, the Spanish isolate M116 of *Watermelon mosaic virus* (WMV), the Spanish C16 isolate of *Zucchini yellow mosaic virus* (ZYMV) and the Spanish M 730 (Song type) isolate of *Cucumber mosaic virus* (CMV). The first three viruses were maintained in *C. pepo* cv. Diamant, and the last one in *C. melo* cv. Amarillo.

Assay plants were kept in an insect-proof glasshouse or growth chambers under controlled environmental conditions (22–24 °C temperature, 60% relative humidity, 16 h light) in Malaga, and in growth chambers using similar conditions in Madrid.

For whitefly transmissions, virus-free *B. tabaci* colonies were maintained on healthy melon plants (*C. melo* cv. Amarillo) in an insect-proof glasshouse (20–32 °C, 45–85% RH) with artificial light supplemented when needed. Whitefly inoculation of test plants with CVYV was performed essentially as described for CYSDV by Marco et al. (2003).

cDNA synthesis, cloning and sequencing

All standard DNA and RNA manipulations were carried out as described by Sambrook and Russell (2001). RT-PCR based strategies were used to clone DNA fragments complementary (cDNAs) to the genomic RNA of CVYV-AILM. Primers were de-

signed using the information of the characterized virus isolate from Israel CVYV-Isr (Lecoq et al., 2000), and from the CVYV-AILM sequence once available. To prepare clone pLMAM48 (see below), the specific oligonucleotides 5′-GTCAG-CCGTCAACTGTGGTGG-3' (MA162) and 5′-ACATTAGCGGCAAGTGTC-TG-3′ (MA163) were used as primers in RT-PCR reactions in which total RNA from CVYV-AILM infected plants was used as template. Primers MA162 and MA163 corresponded respectively to positions 14–34 and complementary to positions 1531–1550 in the CVYV sequence reported by Lecoq et al. (2000). The amplified cDNA fragment (1.5 Kb in length) was gel-purified, ligated into pGEM-T vector System II (Promega, Madison, WI, USA), cloned in *E. coli* DH5α cells and sequenced. To prepare clone pLMCP (see below), oligo(dT) and the specific oligonucleotide 5′-GAATTCACCAAGGCAGA-CGACATTGAGAAAG-3′ (MA228) were used as primers in similar RT-PCR reactions. The sequence of oligonucleotide MA228 was based on the previously determined sequence of the pLMAM48 cDNA insert (with three mismatches compared to the sequence reported by Lecoq et al., 2000, positions 601–619), and it was designed to incorporate an *Eco*RI restriction site and a spacer (underlined) for subcloning purposes. As for pLMAM48, the amplified cDNA fragment was also gel-purified, ligated into pGEM-T vector System II (Promega), cloned in *E. coli* DH5α cells and sequenced. For expression of recombinant CP, after restriction digestion of pLMCP with *Eco*RI (upstream the spacer and CP sequence) and *Spe*I (downstream the 3′ non-coding region of CVYV), the resulting 1.3 Kb fragment was purified and cloned into plasmid pMAL-2c (New England Biolabs, Beverly, MA, USA) previously digested with *Eco*RI and *Xba*I (compatible with *Spe*I). The construct generated was named pMAL-CVYV-CP, and its sequence was determined.

Detection of viral RNA in dot-blots and tissue prints

Total RNA extracts were prepared from leaf tissues of infected and healthy plants (Celix et al., 1996). For detection of viral RNA in dot-blots, 1 μl of total RNA extracts was spotted onto positively charged nylon membranes (Roche Diagnostics GmbH, Mannhein, Germany). For detection of viral RNA in tissue prints (Mas and Pallas, 1995), petioles were cut with a razor blade and, immediately after cutting, the cross-sections were blotted onto positively charged nylon membranes (Roche Diagnostics). CVYV RNA on membranes was detected by molecular hybridization using a digoxygenin-11-UTP-labelled RNA probe synthesized by *in vitro* transcription from the plasmid pLMAM48. Plasmid pLMAM48 was linearized with *Apa*I for transcription of negative sense RNA using SP6 RNA polymerase (Promega). Prehybridization and hybridization of membranes were carried out at 65 °C in standard buffer (5× SSC, 0.1% *N*-Lauroylsarcosine, 0.02% SDS, 2% Blocking Reagent (Roche Diagnostics)) containing 50% formamide. Membranes were washed for 15 min, once at room temperature in 2× SSC and twice at 65 °C in 0.1× SSC. Chemiluminescent detection was carried out using the reagents and protocols supplied by the DIG-labelling and detection kit (Roche Diagnostics).

Expression of recombinant MBP-CP CVYV and proteolytic separation of the viral CP

Plasmid pMAL-CVYV-CP, which contained a copy of the CP gene of CVYV fused in frame after the maltose binding protein (MBP), was transformed into *E. coli* BL21(DE3) cells. Overnight cultures maintained at 25 °C were used to initiate a larger culture for growth at the same temperature and to induce expression of the fusion protein following supplier-provided protocols. Once the OD (600 nm) of the culture reached a value between 0.5 and 1.0, isopropyl *ß*-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM to induce expression of the recombinant protein. After 3 h, cells were centrifuged at 4 °C and resuspended in lysis buffer (10 mM Tris–HCl pH 7.8, 200 mM NaCl, 1 mM dithiothreitol (DTT), 0.25% polyoxyethylenesorbitan monolaurate (Tween-20), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ethylendiaminetetraacetic acid (EDTA)) prior to deep freezing at −80 °C. After thawing, lysis was achieved by adding Lysozyme (2 mg/ml) and shacking during 30 min on ice, followed by five cycles of 30 s sonication. Following a clarification step (30 min centrifugation at 9000 × g), the supernatant was passed through an amylose resin column equilibrated in column buffer (10 mM Tris–HCl pH 7.8, 200 mM NaCl, 1 mM DTT,

0.25% Tween-20). Elution of retained products was performed after several washings, adding column buffer supplemented with 10 mM maltose. Fractions recovered were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify the expected 82 kDa protein band corresponding to the MBP-CP fusion. To proteolytically separate the MBP (42 kDa) and the CP of CVYV (40 kDa), the factor Xa protease (Roche Diagnostics) was added to the eluted product from the column in a ratio around 1:25 (protease:substrate), and the reaction incubated at 4 °C during 2 days, and subjected to SDS-PAGE analysis to evaluate the digestion.

Preparative SDS-PAGE, electroelution, immunization of rabbits and obtention of antisera

Both proteins, the expressed fusion MBP-CP and the proteolytically separated CP, were electrophoresed through standard 4–12% SDS-PAGE gels and stained with a 0.25% R-250 Coomasie brilliant blue solution in water. The protein bands were excised, and after destaining in water, the proteins were electroeluted from the gel matrix in an Electroeluter apparatus (Model 422 from Bio-Rad Laboratories, Hercules, CA, USA) following provider instructions. The electroeluted proteins were concentrated in Centricon YM-30 columns (Millipore Corporation, Bedford, MA, USA) and the elution buffer was substituted with 0.2 M $NaHCO_3$, 0.02% SDS. The products were dried in a Speed-Vac Concentrator (Savant), and resuspended in sterile phosphate-buffered saline (PBS) solution.

Purified proteins were mixed (1:1) with Freund complete adjuvant (Sigma-Aldrich, St Louis, Missoury, USA) and injected subcutaneously in the neck of New Zealand female rabbits, using doses ranging between 80 and 350 μg of protein. Five immunizations were performed at 3 weeks intervals. Check bleedings were drawn 8 days after the fourth injection. The animals were sacrificed 8 days after the fifth immunization to collect their blood. Sera were separated from blood samples by centrifugation after incubations for 30 min at 37 °C and 16 h at 4 °C.

Serological detection of CVYV in plants

Infected and non-inoculated control plants were sampled for detection of virus using a western blot assay (Towbin et al., 1979). Total protein extracts were prepared using Laemmli's extraction buffer, separated by electrophoresis (SDS-PAGE), and transferred to a PVDF membrane (0.45 μm Hybond-P, Amersham Biosciences, Little Chalfont, UK). After a incubation step in blocking buffer (5% low fat milk in PBS), sequential incubations with a 1:1000 (unless otherwise indicated) dilution in blocking buffer of CVYV-specific sera and with a 1:1000 dilution in blocking buffer of peroxidase-labelled goat antirabbit (Sigma-Aldrich) were performed. Colorimetric detection was achieved using a 0.48 mM 4-chloro-1-naphtol (Sigma-Aldrich) solution in PBS with 17% methanol. For chemiluminescent detection, specific antibodies were diluted up to 1:15,000, incubation with the secondary labelled antibody was performed at 1:10,000 dilution, and the ECL Western blotting detection reagents (Amersham Biosciences) were used according to supplier instructions.

Indirect ELISA tests (Clark and Adams, 1977) were performed using CVYV antibodies after cross-absorption to remove non-pathogen antibodies. Briefly, crude sera were diluted 1:10 with PBS, mixed with acetone-extracted powder obtained from healthy cucumber plants (1% w/v), and incubated overnight at 4 °C before centrifugation. ELISA plates (Costar, Corning Incorporated, Corning, NY, USA) were coated for 3 h at 37 °C with plant extracts (1:25 w/v) in carbonate buffer supplemented with 1% low fat milk as blocking agent. Crude extracts were directly used without centrifugation. After coating, standard overnight incubations at 4 °C with immunoabsorbed CVYV antibodies at 1:200, 1:400, 1:1000 and 1:2000 dilutions, and alkaline phosphatase labelled goat antirabbit (Sigma-Aldrich) at 1:1000 or 1:2000 dilutions were performed. The specific substrate 4-nitrophenyl phosphate (pNPP) at 1 mg/ml was used until yellow color development, and absorbance values (405 nm) were measured after several time intervals.

Results

Partial nucleotide sequence of the Spanish CVYV-AILM isolate

Cucumber plants showing the characteristic symptoms induced by CVYV were detected in a

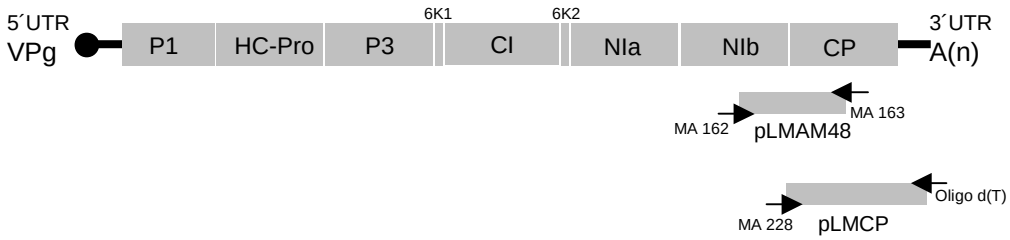
greenhouse of El Ejido (Almeria, Spain) in autumn 2000. Samples were taken from symptomatic apical leaves of one of these plants and used to mechanically inoculate five plants of melon, cucumber and squash. Eight to ten days after inoculation, three melon and two squash plants showed intense vein chlorosis which spread into the tissues adjoining the veins of their younger leaves. Later these plants became stunted and showed bright vein yellowing and generalized chlorosis. Symptomatic melon plants were used as a source of inoculum for three groups of five melon plants which were inoculated using 20, 10 and 5 *Bemisia tabaci* whiteflies, respectively. Ten to thirteen days after inoculation, symptoms were evident in three plants of those inoculated using 20 whiteflies and in one plant of those inoculated using 10 or 5 whiteflies. RT-PCR (Cuadrado et al., 2001b) on symptomatic plants confirmed the presence of CVYV. The isolate infecting the melon plant inoculated using five whiteflies has been called CVYV-AILM and used throughout this study.

Total RNA extracts from melon plants infected with CVYV-AILM were used to generate cDNAs to the 3′-terminal part of the CVYV genome by RT-PCR. cDNAs were cloned in *E. coli* and sequenced for clones pLMAM48 and pLMCP (Figure 1A). These two cDNA clones included an overlapping nucleotide sequence (Figure 1A) which was identical for both of them. Nucleotide sequences derived from pLMAM48 and pLMCP were merged and used in a BLAST search which showed that this sequence shares the maximum sequence identity with other CVYV nucleotide sequences deposited in databases. An analysis of merged sequences revealed the presence of a single open reading frame (ORF) followed by a 3′-untranslated region of 236 nt excluding the poly(A) tail. The deduced aminoacid sequence was aligned with sequences from other members of the *Potyviridae* family (data not shown). According to this alignment, the CVYV-AILM cloned region includes the C-terminal part of the NIb coding region and the complete CP coding region, considered to span the last 359 aminoacid residues coded by the viral ORF (Figure 1B) according to the consensus of NIa protease cleavage sites for other members of the *Potyviridae* family (Garcia et al., 1998). Nineteen amino acid changes were found between the CVYV-AILM and CVYV-Isr (Lecoq et al., 2000) sequences, resulting in a 96.6% identity (Figure 1B). Of these changes, only five were found in the partial NIb sequence corresponding to the C-terminal part of the protein (195 residues), and in particular the characteristic GDD motif of RNA dependent RNA polymerases was conserved (Figure 1B, positions 44–46). Comparison of the CP sequence of CVYV-AILM with that of CVYV-Isr showed 14 aminoacid substitutions, 2 in the core and C-terminal regions (Shukla et al., 1994), and 12 in the N-terminal variable portion comprising the 125 first residues of the CP. Interestingly, seven changes of those found in that region are conservative and affected charged aminoacids. The highly conserved DAG triplet motif located near the N-terminus of the CP of most aphid-transmitted potyviruses (Lopez-Moya et al., 1999) is not present in the CVYV CP sequence. The GenBank accession number for partial sequence of isolate CVYV-AILM is AY290865.

Detection of CVYV by RNA hybridization

Melon, cucumber and squash plants were inoculated with CVYV-AILM, CMV, CYSDV and WMV. Total RNA extracts from infected and mock-inoculated plants were used in dot-blot hybridizations to determine the specificity of a cRNA DIG-labelled probe prepared by *in vitro* transcription from the pLMAM48 cDNA clone (Figure 1A). In addition, tissue prints of cross-sections of petioles of infected and mock-inoculated plants were also included in membranes for hybridizations. As illustrated in Figure 2A, signals appeared only when tissue prints or total RNA extracts were from CVYV-infected plants, and no signals appeared when tissue prints or total RNA extracts were from mock-inoculated plants or from plants inoculated with viruses different to CVYV. To determine the sensitivity of the dot-blot detection method, serial dilutions of viral RNA transcribed from pLMAM48 were inserted in membranes and hybridized using the above mentioned probe. Dilutions were performed either in water or in total RNA extracts from healthy plants. As shown in Figure 2B, CVYV RNA detection was possible up to dilutions of 6×10^{-6} μg. In our hands, RT-PCR detection of CVYV RNA using primers described by Cuadrado et al. (2001b) is just 10–100 fold more sensitive than that (data not shown).

A



B

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CVYV-AlLM       QPSTVVDNTLILMLVVEYCKSWHFKQSGVKMEFKYMCNGDDLIINAPDREMSIIQSTFKN     65
CVYV-Isr        QPSTVVDNTLILMLVVEYCKSWHLKQSGIKMEFKYMCNGDDLIINAPDKEISIIQSTFKN     65
                ************************:****:******************:*:*********

CVYV-AlLM       LFKECGLNYDFDDLHDSIEDVEYMSHHFVLREGFYIPKLSKERLVAILEWERSDELFRTR    125
CVYV-Isr        LFKECGLNYDFDDLHDSIEDVEYMSHHFVLREGFYIPKLSKERLVAILEWERSDELFRTR    125
                ************************************************************

CVYV-AlLM       SALNAAYIESFGYDDIHWEIERFAAYWANLKGVKNVLMSEDHVRKLYLDENFELTDEIVQ    185
CVYV-Isr        SALNAAYIESFGYDDIHWEIERFAAYWANLKGVNNVLMSEDHVRKLYLDENFELTDEIVQ    185
                ********************************:***************************
                          NIb|CP
CVYV-AlLM       TLSPASFEFGYVELQADDIEKEAIEQEIEKLRNEWKANGPSRTVSNYEARKRQTPIAAKV    245
CVYV-Isr        TLSPASFEFGYVELQADGIDKEAIEQEIEKLRNDWKTNGPSRTVSNYEARKKQTPIAAKV    245
                ****************.*:*************:**:**************:********

CVYV-AlLM       DELLKQLKEAGVETLKRPCGQPNADEDKKENSNSNWTGESDEEDEGKKKRMPLRGGGNMM    305
CVYV-Isr        DELLKQLKEAGVETLKRPCGQPNADEDKKESSNSNWTGESEDEDEEKKKKMPLRGGGKMM    305
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CVYV-AlLM       KRDDVDKIPTNAMEFKRDFKPARASRTSYIWIPRSQRDNLTPDVVKNFLAYIPPSQAIDN    365
CVYV-Isr        KRDDVDKIPTNAMEFKKDFKPARASRTSYIWIPRSQRDNLTPDVVKNFLAYIPPSQAIDN    365
                ****************:*******************************************

CVYV-AlLM       QMASGSQVENWAMRTASAYGVTIQQFYETVLPAWIVNCIVNGTSDERKTETVWRAVELNA    425
CVYV-Isr        QMASGSQVENWAMRTASAYGVTIQQFYETVLPAWIVNCIVNGTSDERKTETVWRAVELNA    425
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CVYV-AlLM       QGEDVDDMEYPIEPIYKHALPTMRKIMRNFSSQAILMYQNSVAEGKAFTVKAARNAGYTE    485
CVYV-Isr        QGEDVDDMEYPIEPIYKHALPTMRKIMRNFSSQAILMYQNSVAEGKAFTVKAARNAGYSE    485
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CVYV-AlLM       IEDQWLGIDFLAEAQLSRNQLNIKHQTLAANVSRNRRNLFALAAPGDDGRVNAERHLTTD    545
CVYV-Isr        IEDQWLGIDFLAEAQLSRNQLNIKHQTLAANVSRNRRNLFALAAPGDDGRVNAERHLTTD    545
                ************************************************************

CVYV-AlLM       ASASRHTYGGAMIE                                                  559
CVYV-Isr        ASASRHTYGGAMLE                                                  559
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Figure 1. Partial nucleotide sequence of the Spanish CVYV-AILM isolate. (A) Diagram showing the localization in the potyviral genome of the cDNA clones prepared and sequenced for CVYV-AILM. Arrows indicate the positions of primers used for generation of cDNAs. Designations of primers and clones are indicated below the arrows and the shadowed boxes, respectively. (B) Alignment of deduced aminoacid sequences of the genomic coding regions sequenced for CVYV-AILM and CVYV-Isr (Lecoq et al., 2000). The putative N-terminus of the CP is indicated by a vertical line. Conserved residues (*) and conservative (:) or non-conservative (.) changes are indicated. Numbers follow Lecoq et al., 2000.

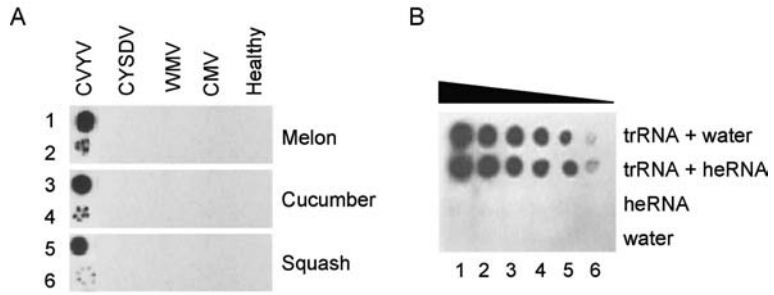

Figure 2. Detection of CVYV by RNA hybridization. (A) Example of an autoradiograph from a membrane containing total nucleic acid extracts of plants (lanes 1, 3 and 5) and tissue prints of petiole cross-sections (lanes 2, 4 and 6) hybridized to detect CVYV RNA. Melon, cucumber and squash plants infected with CVYV-AILM, CYSDV, WMV and CMV, and mock-inoculated (healthy) plants, were included in this analysis. (B) Dot-blot hybridization of transcribed viral RNA from the pLMAM48 cDNA clone to analyze the sensitivity of CVYV RNA detection. Transcribed RNA (trRNA) were diluted either in water or in total nucleic acid extracts from healthy melon plants (heRNA) in a 1:10 dilution series, starting from 6×10^{-1} μg (columns 1–6).

The probe derived from clone pLMAM48 is being used for routine diagnosis of the presence of the virus in cucurbit field samples from Almeria, Malaga and Murcia (Spain). Whenever a contrasting detection method was used (e.g. RT-PCR), it was observed that this probe, in either of both hybridization methods described above, consistently detected CVYV in all samples (data not shown).

Expression and immunogenicity of the recombinant CVYV CP

Extracts of *E. coli* BL21(DE3) cells which had been transformed with plasmid pMAL-CP-CVYV after induction of expression contained a protein band with 82 kDa corresponding to the MBP-CP fusion. After affinity chromatography, the product was purified to near homogeneity, as observed in the SDS-PAGE analysis. The yield achieved was estimated between 10 and 15 mg of purified protein per liter of culture. Following proteolytical digestion with factor Xa protease, the 82 kDa product was excised into two proteins of 42 and 40 kDa, corresponding to the MBP and the CP of CVYV respectively. Both proteins, the fusion product MBP-CP and the CP, were able to raise immunogenic responses in rabbits, generating CVYV specific polyclonal antibodies.

Serological detection of CVYV

Cucumber plants inoculated with CVYV showing clear symptoms were used to test the ability of the polyclonal antibodies to detect the presence of the virus. Western blot analysis of total proteins extracted from infected cucumber plants showed the specific detection of a 40 kDa band, not present in extracts from uninfected control plants (Figure 3A, lanes 1 and 2). Both antisera, the one from the rabbit immunized with the CP CVYV (Figure 3) and the one from the rabbit immunized with the fusion MBP-CP CVYV (data not shown) reacted with the same product. Optimal concentration of sera was determined by using different dilutions, finding that the best detection was achieved at 1:1000 using chloronaphtol colorimetric detection. More concentrated dilutions resulted in the occasional appearance of unexpected unspecific reactions without improving the level of detection, and lower dilutions resulted in weaker signals. For chemiluminescent ECL detection, dilutions up to 1:15,000 were still adequate.

Indirect ELISA assays showed consistent positive detection of CVYV, with absorbance readings at least two times higher in samples from infected than those from uninfected plants. The best detection was obtained with 1:1000 dilution of immunoabsorbed antiserum and 1:2000 dilution of secondary GAR-AP, because in these conditions controls gave lower readings, and thus the corrected ratio infected/healthy reached values in the range of 4–5. An example of readings obtained after 4 h of substrate incubation (A_{405}, mean of four independent wells coated with the same extracts) showed that the extract from a CVYV-infected cucumber plant reached 1.5055 ± 0.0719,

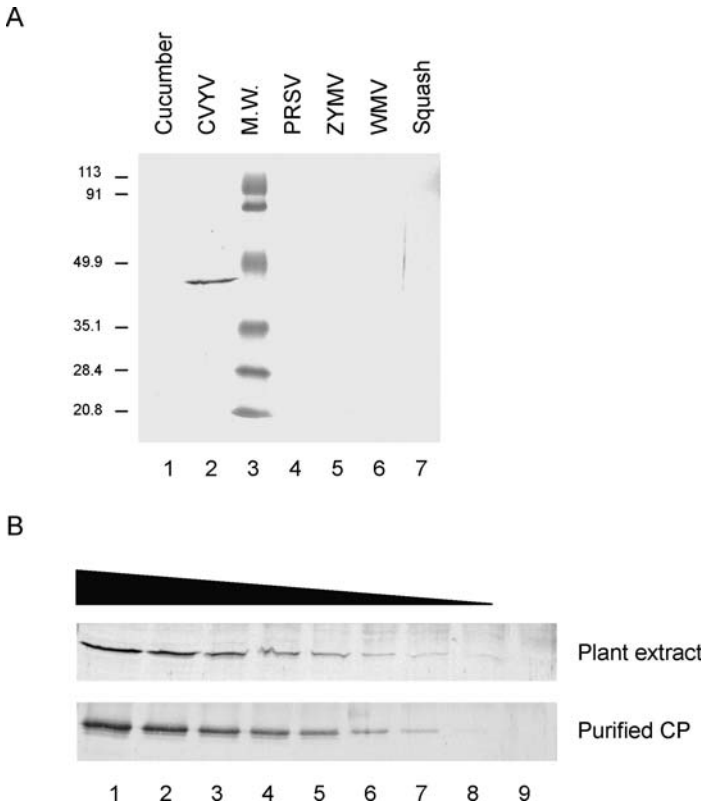

Figure 3. Serological detection of CVYV. (A) Western blot analysis of uninoculated (lane 1) and CVYV-infected cucumber plants (lane 2). Lanes 4–7 correspond to squash plant extracts uninoculated (lane 7) or infected with PRSV (lane 4), ZYMV (lane 5) and WMV (lane 6). Molecular weight markers (Bio-Rad) were loaded (lane 3) and are shown on the left. (B) Sensitivity of CVYV CP detection. Crude 1:5 (w/v) infected plant extracts (above) and 100 ng of purified recombinant CP (below) were diluted in 1:2 dilution series, using PBS, and analyzed by western blot (lanes 1–8). Twenty microliters of each dilution of infected plant were loaded per well. Lanes 9 were loaded with buffer.

while an uninfected plant extract gave 0.563 ± 0.0272, which represents a 4.88 ratio between corrected values after subtracting blank readings (0.32025 ± 0.0363).

The presence of viral CP was investigated in different organs and tissues of infected cucumber plants using the antibodies in western blot experiments. Virus was detected in all organs tested, including roots, stems, leaves, petioles, tendrils, flowers and fruits (data not shown).

Specificity and sensitivity of detection with antibodies

Infected cucurbits plants were submitted to western blot analysis. Three potyviruses (PRSV, WMV-II and ZYMV) infecting squash plants, and one cucumovirus (CMV) and one crinivirus (CYSDV) infecting melon plants were tested, along with the corresponding uninfected plants of each species. The CVYV antibodies did not recognize any of the five cucurbit viruses tested, nor there were any detectable reaction with the extracts from uninfected control squash or melon plants, while the CVYV CP was consistently detected only on CVYV-infected cucumber plants that were processed in parallel. These results indicated that the antisera produced was specific for CVYV, as shown in Figure 3A with the three potyviruses (lanes 4–6).

To check if different CVYV isolates could be detected by the antibodies, a western blot analysis was performed. This showed that a different Spanish isolate from Almeria and the previously characterized CVYV-Isr from Israel (Lecoq et al., 2000) gave similar reactions to those of the homologous CVYV-AILM which was used to immunize the animals.

Serial dilutions of total protein extracts (1:5 w/v) from CVYV-infected plants were analyzed to determine the sensitivity of detection. Dilutions up to 1:320 w/v were positive in western blots using colorimetric substrate for the presence of the virus (Figure 3B). Western blot analysis was used also to find the limit of detection of gel-purified recombinant CP protein, finding that 1.5 ng of protein was positively detected (Figure 3B). Dilutions were performed using PBS buffer.

Discussion

A partial sequence of the Spanish CVYV-AlLM isolate covering the entire CP and part of the NIb region of the virus was obtained. Comparison with the previously published sequences from a Middle East isolate (CVYV-Isr; Lecoq et al., 2000) showed a relatively high degree of conservation, in spite of their different geographical origin. The Spanish isolate has an unknown point of provenance, and further information will be needed to determine if the observed conservation reflects the variability of virus populations in different areas and within the same region.

The analysis of the obtained sequence showed that the N-terminus of the CP contained a segment with a high number of charged residues, a feature that is conserved among several other members of the *Potyviridae* family (Shukla et al., 1994). Interestingly, this segment of the CP sequence included most of the differences at the amino acid level between the sequence of the CVYV-AILM isolate and the other previously reported sequence. Within the complete CP region, twelve changes out of fourteen were found in the N-terminal part, and of them seven were conservative changes affecting charged residues. The involvement of the equivalent region of the CP of several potyviruses in important aspects of their life cycles has been demonstrated, and functions such as movement of the virus in the plant, aphid transmissibility and accumulation could be affected by mutations in this region (Dolja et al., 1994; Andersen and Johansen, 1998; Andrejeva et al., 1999; Lopez-Moya et al., 1999). In particular, the importance of net charge in the variable N-terminus of the CP of potyviruses has been mentioned earlier to affect virus movement (Lopez-Moya and Pirone, 1998). Our observation that the majority of aminoacid substitutions in the CVYV CP N-terminus resulted in conservation of the charge suggests that this region might be also related with one or more of these important functions during the life cycle of CVYV.

Apart from the complete nucleotide sequence of the type member of the *Ipomovirus* genus, *Sweet potato mild mottle virus* (SPMMV) (GenBank Z48058), only a partial sequence of a *Cassava brown streak virus* isolate (CBSV) is available (GenBank AY007597). Unfortunately, the CBSV partial sequence does not comprise the complete CP coding region of this virus. The insufficient sequence information available makes it difficult to identify putative genus-specific features. However, a few aspects could be mentioned. First, the CPs of ipomoviruses share many distinctive characteristics with the CPs of other genera in the *Potyviridae* family, mainly in the core region. The N-terminus is the most variable in length and sequence, and no DAG-like motif was observed. This motif is characteristic of aphid-transmitted potyviruses (Lopez-Moya et al., 1999), and thus its presence or absence might be one of the molecular factors that govern vector specificity. No equivalent motif could be identified in this region in whitefly-transmitted ipomoviruses. In fact, there is no evidence of the dependency of a helper component factor during the insect transmission of ipomoviruses as it is needed for aphid-transmitted potyviruses (Pirone and Blanc, 1996). Further investigations are needed to analyze this aspect.

The CVYV-AILM cDNA clones obtained allowed us to prepare good diagnostic systems for this virus. The specificity of the detection methods was tested including several of the most common cucurbit-infecting viruses that are present in crops of the area where CVYV is spreading (Luis-Arteaga et al., 1998; Marco et al., 2003; Sevik and Arli-Sokmen, 2003; Aranda and Lopez-Moya, unpublished). Our results indicated that the RNA probe and the antibodies detected the presence of CVYV without any detectable cross-reaction with other viruses. As mentioned above, the comparison of sequences of the two CVYV isolates from which partial information is available suggests a high conservation in spite of their different origin, and therefore at this point the tools developed seemed to allow a sensitive and specific detection of the virus. Indeed, the positive serological detection of isolates from very distant regions (Spain and Israel)

substantiates that, although more information on virus variability will be highly desirable to confirm these aspects.

The detection tools described have many practical applications. For instance, they could be used in strategies to control the disease, such as surveillance of plant material to avoid the entrance of the virus in regions where it has not been found, or to eradicate reservoirs that could act as sources of inoculum in those areas where the virus is already present. Apart from these immediate uses, also basic scientific studies can benefit from the availability of these detection tools, such as investigations on distribution, accumulation and movement of the virus in the plant, or analysis of the structural properties of the virus particles, including procedures for purification and studies on insect transmissibility. In particular, the CVYV viral particles appear to be unusual because of the difficulties in their isolation. We have found that the particles tend to aggregate, and therefore they are lost easily during the early clarification steps, as observed with the assistance of the specific antibodies developed (data not shown). Alternatives to improve the purification protocol are being pursued. These peculiarities of behavior of the viral particles might be related with the considerable difficulties observed to detect the presence of the virus in crude extracts, for instance in ELISA tests. Alternatively, since our antisera were raised against recombinant proteins, we cannot rule out that they recognized epitopes not exposed in the folded CP protein assembled in particles. This could explain the fact that a denaturing step, as done in western blot analysis, might be needed to disrupt particles and expose epitopes. In any case, further experimentation will be needed to resolve this point.

The detection methods would also serve to study the movement and distribution of virus within the plant. Our preliminary observations suggests that the virus was present in most organs and tissues of inoculated cucumber test plants, and their accumulation was maintained at high levels for substantial period of time. However, this might not be the situation in other conditions, for instance in field crops. Indeed, a recent report of a rapid detection method based in hybridization with a cDNA probe suggests that CVYV shows irregular distribution within different host plants (Rubio et al., 2003). The antibodies described here could be helpful to determine if the distribution of the virus varies in different hosts, developmental stages and environmental conditions. As before, the development of routine diagnostic protocols should take into account all these peculiarities of CVYV.

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