Molecular characterization and serological diagnosis of the new emerging *Cucurbit chlorotic yellows virus* in Taiwan

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Abstract


*Crinivirus*, a genus of the family *Closteroviridae*, has large positive-sense single-stranded RNA genomes encapsidated in long flexuous virions and is transmitted by whitefly.  The virus bipartite particles of 650-900 × 12 nm comprise two genome segments, denoted RNA1 and RNA2.  *Criniviruses* infect several important crops worldwide, causing symptoms of interveinal chlorosis, yellowing and brittleness of leaves accompanied by severe yield losses.  In April 2009, typical crinivirus-induced symptoms were observed on melon (*Cucumis melo* L.) plants in Yunlin County, Taiwan.  The degenerate primers designed for *Closterovirus* and *Crinivirus* were used in nested reverse transcription-polymerase chain reaction (RT-PCR) to amplify a 0.5 kb DNA fragment from the symptomatic melon tissues.  Subsequently, the amplified DNA fragment was determined to share 100% identity with the reported nucleotide (nt) sequence of *Cucurbit chlorotic yellows virus* (CCYV), a new *Crinivirus* species found in Japan.  In this study, an isolate of crinivirus denoted as *Crinivirus-TW* was obtained from a diseased melon plant and maintained in *Nicotiana benthamiana* by whitefly transmission.  Furthermore, the complete nt sequence of *Crinivirus-TW* RNA2 was determined as 8,041 nt in length containing eight open reading frames (ORFs), including P5, Hsp70h, P6, P60, P9, CP, CPm and P26.  Sequence analyses revealed that the RNA2 sequence of *Crinivirus-TW* shares 99% nt identity with that of the original Japan isolate of CCYV.  Thus, *Crinivirus-TW* was identified as an isolate of CCYV, renamed CCYV-TW.  Phylogenetic analyses of the Hsp70h, P60, CP, CPm and P26 among *Crinivirus* species revealed that CCYV-TW is closely related to *Lettuce chlorosis virus* (LCV), *Bean yellow disorder virus* (BYDV) and *Cucurbit yellow stunting disorder virus* (CYSDV).  The recombinant CP of CCYV-TW expressed by the bacterial pET expression system was used as an immunogen for production of monoclonal antibodies (MAbs).  The MAbs prepared were successfully used to detect CCYV in diseased plants by western
blotting and to examine the accumulation of virus in phloem cells by tissue blot immunoassay (TBIA). Moreover, primer pair Crini-hsp70h-f/Crini-hsp70h-r, designed from the Hsp70h gene, coupled with primer pair mt-F2/mtR1, designed from the plant mitochondrial NADH dehydrogenase (nad5) gene, used in multiplex one-step RT-PCR was developed as an effective detection method and applied in field survey. A number of 253 diseased melon samples collected from central Taiwan during February to June in 2010 were tested by multiplex one-step RT-PCR. An increased incidence of 3.8% to 100% was noticed. Our results indicated that CCYV has become an important threat for the production of cucurbits in Taiwan, and it should be majorly concerned.

Keywords: Crinivirus, Cucubit chlorotic yellows virus (CCYV), melon

Introduction

Cucurbits are economically important crops in many countries, and the virus disease is one of the major limiting factors for their productions. So far, at least 40 different virus species have been reported to naturally infect cucurbits all over the world (10, 37). Melon (Cucumis melo L.), cucumber (C. sativus L.), watermelon [Citrus lanatus (Thunb.) Matsum. & Nakai], sponge gourd [Luffa cylindrica (L.) Roem.], bitter gourd (Mormodica charantia L.), bottle gourd (Lagenaria siceraria Standl.) and wax gourd (Benincasa hispida Cogn.) are important cucubit crops planted more than 23,000 ha each year in Taiwan (Yearly Report of Taiwan’s Agriculture, 2008). Eleven virus species belonging to five genera had been reported to infect cucurbits in Taiwan. The members of the genus Potyvirus, including Papaya ringspot virus watermelon type (PRSV-W) (23, 38), Zucchini yellow mosaic virus (ZYMV) (27, 41) and Melon vein-banding mosaic virus (MVbMV) (28), are considered the most widely distributed viruses. Watermelon silver mottle virus (WSMoV) (66) and Melon yellow spot virus (MYSV) (8, 9, 48) belonging to the genus Tospovirus, Cucumber green mottle mosaic virus (CGMMV) (23) belonging to the genus Tobamovirus, Cucumber mosaic virus (CMV) (6, 23) belonging to the genus Cucumovirus, Squash leaf curl Philippines virus (SqLCPhV) (40) belonging to the genus Begomovirus and Melon aphid-borne yellows virus (MABYV), Cucurbit aphid-borne yellows virus (CABYV) and Suakwa aphid-borne yellows virus (SABYV) belonging to the genus Polerovirus (14, 36) are also prevailing.

In April 2009, virus-like symptoms showing yellows, chlorotic spots, chlorosis and brittleness on leaves of melon plants were found in Lunbei Township, Yunlin County (29). The special symptoms was similar to those caused by Cucurbit yellow stunting disorder virus (CYSDV), a member of the genus Crinivirus, as described previously (63). Large populations of silverleaf whiteflies (Bemisia argentifolii Bellows & Perring) were observed on leaves of the affected melon plants. Based on the symptomatology, the virus morphology and the suspected insect vector, a crinivirus was suspected as the causal agent. Degenerate primers designed for Clasterovirus and Crinivirus (15) were used in nested RT-PCR to amplify a 0.5 kb of DNA fragment from total RNAs extracted from the symptomatic melon tissues. Subsequently, the amplified DNA fragments were determined to share 100% nucleotide (nt) identity with the partial sequence of Hsp70h gene of Cucurbit chlorotic yellows virus (CCYV), a new Crinivirus species recently discovered in Japan (47). Thus, the newly emerging virus was considered as a CCYV isolate.

CCYV is a member of the genus Crinivirus in the family Clasteroviridae that transmitted by silverleaf whiteflies in a noncirculatire and nonpropagative semi-persistent manner (47). All members of the family Clasteroviridae have a large positive-sense single-stranded (ss) RNA genome ranging from 15 to 20 kb, which are encapsidated in long flexuous filamentous virions of 700-2000 nm × 12 nm (32, 43). Crinivirus comprises two RNA segments, denoted RNA1 and RNA2, encapsidated separately (33). Two open reading frames (ORFs), ORF1a
and ORF1b, associated with virus replication exist in RNA1. ORF1a encodes a polyprotein containing a papain-like protease (P-Pro), a methyltransferase (MT) and a helicase (HEL), and ORF1b encodes an RNA-dependent RNA polymerase (RdRp) \(^{(32, 46)}\). RNA2 contains the hallmark gene array of the family **Closteroviridae** to encode a small hydrophobic protein, a heat shock protein 70 homologue (Hsp70h), a 60 kDa protein (P60), the major coat protein (CP) and the minor CP (CPm) \(^{(32)}\). Hsp70h and P60 are highly conserved within the family **Closteroviridae**. They are physically associated with virus particles involving in virion assembly \(^{(45, 56)}\) and virus movement \(^{(3, 4, 49, 52, 58)}\). CP encapsulates viral genomic RNA to form virion “body”, whereas the CPm only binds to the tip of one end of the virion to form a “rattlesnake tail” \(^{(1, 53, 56)}\). The demarcation criteria of **Crinivirus** species includes particle size, vector specificity, host range, serological specificity, genome structure and organization, cytopathological features and the difference of more than 25% of amino acid (aa) sequences of the RdRp, CP and Hsp70h \(^{(33)}\). To date, 14 species, such as *Abutilon yellows virus* (AbYV) \(^{(42)}\), *Bean yellow disorder virus* (BYDV) \(^{(44)}\), *Beet pseudo-yellows virus* (BPYV) \(^{(12, 16)}\), *Blackberry yellow vein-associated virus* (BYVaV) \(^{(55, 59)}\), *CCYV* \(^{(47)}\), *CYSV* \(^{(5)}\), *Lettuce chlorosis virus* (LCV) \(^{(19, 62)}\), *Lettuce infectious yellow virus* (LIYV) \(^{(17, 34, 35)}\), *Potato yellow vein virus* (PYVV) \(^{(54)}\), *Sweet potato chlorotic stunt virus* (SPCSV) \(^{(25, 26, 50, 60)}\), *Tomato infectious chlorosis virus* (TICV) \(^{(18, 61)}\), *Tomato chlorosis virus* (ToCV) \(^{(64)}\), *Strawberry pallidosis-associated virus* (SPaV) \(^{(57, 58)}\) and *Diodia vein chlorosis virus* (DVCV) \(^{(30)}\), have been reported.

In this study, the newly emerging cucurbit-infesting CCYV in Taiwan was further identified by sequence determination and analyses of the genomic RNA2. Based on the determined sequence, primer pairs are designed and CP monoclonal antibodies (MAbs) are also prepared for diagnosis of CCYV in field. Furthermore, the occurrence of CCYV in Taiwan is also discussed.

**Materials and methods**

**Virus isolation**

The diseased melon leaves collected from a field in Lunbei Township, Yunlin County were cut into small pieces (1.5 × 1.5 cm\(^2\)) and placed in a 50 ml-centrifuge tube, and 20 to 30 individuals of virus-free silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring) were transferred into the centrifuge tube for a 24 hr acquisition access period (AAP). Thereafter, whiteflies were transferred into a setup box and fixed on leaves of a healthy *Nicotiana benthamiana* plant for 72 hr inoculation access period (IAP). After IAP, the whiteflies and the leaf tissues of the inoculated *N. benthamiana* plant were collected in a 2 ml-microcentrifuge tube for total RNA extraction using Total RNA Miniprep Purification Kit (GMbiolab, Taichung, Taiwan). RT-PCR was conducted using primer pair Crini-hsp70h-f/Crini-hsp70-r \(^{(29)}\) to confirm the presence of virus in whiteflies and the inoculated plant.

**Determination of viral genomic sequence**

All reported RNA2 sequences of criniviruses were obtained from the database of National Center for Biotechnology Information (NCBI), and the accession codes corresponding to the individual sequences are listed in Table 1. Multiple alignments of the hallmark genes of criniviral RNA2 sequences were conducted by ClustalW program of Biology Workbench, San Diego Supercomputer Center (SDSC) (http://workbench.sdsc.edu/) to estimate consensus sequences for design of degenerate primers. Specific primers were also designed from the determined sequences. Sequences of the degenerate and specific primers used in this study are listed in Table 2.

Total RNAs were extracted from leaf tissues of the virus-infected plants by Plant Total RNA Miniprep Purification Kit (GMbiolab). Reverse transcription (RT) was performed using 1 μg of total RNAs mixed with 10 nM individual primer pairs and 25 U Moloney murine leukemia virus (MMLV) reverse transcriptase (GMbiolab). Mixtures were incubated at 42°C for 50 min to synthesize the first strand cDNAs, and then the reaction was inactivated by heating at 72°C for 15 min. Subsequently, cDNAs were heated at 94°C for 2 min and mixed with 2.5 U EX *Taq* DNA polymerase (Takara, Shiga, Japan). Polymerase chain
reaction (PCR) was performed by 40 cycles of strand separation at 94°C for 30 sec, annealing at 48-60°C for 30-60 sec depending on the properties of primers, and synthesis at 72°C for 1 min, and a final reaction at 72°C for 7 min. The amplified DNA fragments were analyzed by 1% agarose gel electrophoresis and then eluted from gels by Micro-Elute DNA Clean/Extraction Kit (GMbiolab). The eluted PCR products were ligated to the pCR2.1-TOPO vector by TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and transformed into E. coli DH5α competent cells as the manufacturer’s instruction. Sequences were determined by ABI 3730XL automatic DNA sequencing system (Perkin-Elmer Applied Biosystems, Foster City, CA) performed by Mission Biotech Company (Taipei, Taiwan).

The determined nt sequences were assembled by ContigExpress program of Vector NTI version 11 (Invitrogen). The aa sequences were translated from the assembly nt sequence by the SIXFRAME program of Biology Workbench, SDSC. The comparisons of nt and aa sequences of the individual coding regions were performed by the AlignX program of the Vector NTI.

**Phylogenetic analysis**

Input data of multiple aa sequence alignments obtained from the ClustalW program were changed to the phylip format. Phylogenetic analyses were conducted using Phylip 3.68 (University of Washington, WA). Bootstrapping was produced 1,000 repeats to generate multiple reassembled data sets by the Seqboot program of Phylip 3.68. Distance matrix of aa sequences was produced by the Protdist program of Phylip 3.68 under PAM matrixes of Dayhoff model (13). Phylogenetic branches were set by the Neighbor program of Phylip 3.68 using Neighbor-Joining method (51). Finally, phylogenetic trees were produced by the Consense program of Phylip 3.68.

**Virus detection in multiplex one-step RT-PCR**

Symptomatic samples of melon (Cucumis melo L.), cucumber (C. sativus L.), watermelon [Citrullus lanatus (Thunb.) Matsum & Nakai], bottle gourd (Lagenaria siceraria Standl.), wax gourd (Benincasa hispida Cogn.) and squash (Cucurbita moschata Duchesne) showing chlorosis and yellows on leaves collected from natural fields in central
Table 2. The primers used for detection and sequencing of the RNA2 of the new emerging crinivirus in Taiwan

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dC-TW-RNA2-1136</td>
<td>F</td>
<td>CGCCT(A/G)CCACTAAA(A/C)T(A/G)AACTGG(T/C)(T/G)TC</td>
</tr>
<tr>
<td>dC-TW-RNA2-3439</td>
<td>F</td>
<td>TGGGTTGT(A/T)(C/A)AT(A/T)CAC(A/T/C)GAA(A/G)(A/T/G)GA</td>
</tr>
<tr>
<td>dC-TW-RNA2-4459c</td>
<td>R</td>
<td>AGTTTGAAGAA(A/G)TC(A/G)AATCTCAATCC-3′</td>
</tr>
<tr>
<td>dC-TW-RNA2-5083</td>
<td>F</td>
<td>CAGA(A/T)CACATG(A/G)ATCC(A/T)(A/G)(A/C)GAAAA(T/C)T</td>
</tr>
<tr>
<td>dC-TW-RNA2-5629c</td>
<td>R</td>
<td>C(T/C)TTTCAAAGC(T/C)TGGCA(T/C)TTCA TCA</td>
</tr>
<tr>
<td>dC-TW-RNA2-6080c</td>
<td>R</td>
<td>T(T/G)AT(T/C)TT(T/C)(A/T)/(C/A)TAT(T/C)TGAAACCATT</td>
</tr>
<tr>
<td>Specific primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-TW-RNA2-1</td>
<td>F</td>
<td>GGAAATTATCCACCGTTTTCCCCGAG</td>
</tr>
<tr>
<td>C-TW-RNA2-1248c</td>
<td>R</td>
<td>CGAAAAAGTGGTACCAAAATTCCAAA</td>
</tr>
<tr>
<td>C-TW-RNA2-1504</td>
<td>F</td>
<td>GTCAAATTTGACTGGAATTGATCGT</td>
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<tr>
<td>C-TW-RNA2-1593c</td>
<td>F</td>
<td>TAGTCTGACCAAGTGCTCAACGTAC</td>
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<tr>
<td>C-TW-RNA2-2454</td>
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<td>GAATGTATATGAAGGGTCTGATCCA</td>
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<td>C-TW-RNA2-3638c</td>
<td>R</td>
<td>ATGTGTTGTATATCCCTCACTATC</td>
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<tr>
<td>C-TW-RNA2-4254</td>
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<td>C-TW-RNA2-5484</td>
<td>F</td>
<td>ATGAGCGACCATACATCTACAGGC</td>
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<tr>
<td>C-TW-RNA2-5745</td>
<td>F</td>
<td>CTGACAAGTATATGTGTTGTCAATC</td>
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<tr>
<td>C-TW-RNA2-6944</td>
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<tr>
<td>C-TW-RNA2-7178</td>
<td>F</td>
<td>GCCCATGGGCGCATATGAGCTTGATGATGAATAG</td>
</tr>
<tr>
<td>C-TW-RNA2-8041c</td>
<td>R</td>
<td>GGCTTACGCTATGCTACTAATGTCC</td>
</tr>
</tbody>
</table>

“F” and “R” represent the primers were designed in forward and reverse directions, respectively.

Taiwan, the townships of Yunlin County, and the southern Taiwan, the townships of Chiayi, Tainan, Kaohsiung and Pingtung counties. The previously reported primer pairs, Crini-hsp70-f/Crini-hsp70-r (29) and CCYV-HSP-F (5′-TGCGTATGTCAATGGTGTATG-3′)/CCYV-HSP-R (5′-ATCCTTCGCAGTGAAAACC-3′) (21) for Hsp70h were used to detect the virus. Primer pair, mt-F2 (5′-GCTTTCTGGGCGCTTCTGTTGCATA-3′)/mtR1 (5′-ATCCTCCAGTCACCAACCATTGGCAT-3′), designed from the plant mitochondrial NADH dehydrogenase (nad5) gene (39), was used as the internal control. Total RNAs from collected samples were extracted by Plant Total RNA Miniprep Purification Kit (GMbiolab). Virus detection was performed by One-Step RT-PCR Kit (GMbiolab) as the manufacturer’s instruction with modifications. The first strand cDNAs were synthesized at 50°C for 30 min and terminated at 94°C for 2 min. PCR was conducted in 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. The amplicons were examined in 1% agarose gels after staining by SafeView DNA Stain (GMbiolab).

In addition, symptomatic melon samples were collected from fields in Lunbei Township, Yunlin County, Taiwan at a cultivated season for field survey. Multiplex one-step RT-PCR using primer pairs...
Expression of the fusion CP

Total cell proteins were used for evaluating the expression of the fusion CCYV CP by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described previously (31). A 2 ml-bacterial culture, containing antibiotics, kanamycin and chloramphenicol, was incubated at 37°C for 6 hr with shaking at 220 rpm. One milliliter aliquot of the 2 ml-culture was removed and 10 μl of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the aliquot to a final concentration of 1 mM for induction at 37°C for 4 hr with shaking at 220 rpm. Another 1 ml aliquot cultured at the same condition without induction was used as a control. Subsequently, the cells were harvested by centrifugation at 13000 rpm for 3 min. The pellets of total cells were collected and resuspended in 200 μl of 1× PBS (136 mM NaCl, 1 mM KH2PO4, 8 mM Na2HPO4·12 H2O, 2 mM KCl and 3 mM NaNO3) and 100 μl of 2× protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 12% glycerol, 0.01% bromophenol blue and 2% β-mercaptoethanol), and proteins were denatured at 100°C for 3 min. The expressed proteins were observed in 12% SDS-PAGE and confirmed by western blotting using the rabbit antiserum against the histidine tag (RAs-His) (Viogene, Taipei, Taiwan).

Purification of the expressed CP

An 80 ml-culture, containing antibiotics, kanamycin and chloramphenicol, and a 0.8 ml-overnight cultured starter, was incubated at 37°C with shaking 220 rpm until OD600 reaching to 1.0. Thereafter, 100 mM IPTG was added to a final concentration of 1 mM to induce the expression of the recombinant Crinivirus-TW CP that fused with a histidine tag at the N-terminal end. After a 4 hr-induction, bacterial cells were pelleted by centrifugation at 8,000 rpm for 10 min at 4°C. After frozen at -20°C overnight, the pellets were resuspended thoroughly in 8 ml lysozyme buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl containing 300 μl of 250 U lysozyme) and incubated at 37°C for 30 min. The lysates were then sonicated at 100% amplitude on ice for 1 hr. After sonication, the insoluble inclusion bodies were collected by centrifugation at 8,000 rpm for 10 min at 4°C. The pellets were resuspended with binding buffer (500 mM NaCl and 20 mM Tris-HCl, pH 7.9) and centrifuged at 10,000 rpm for 10 min. To repeat this step, centrifugation at 8,000 rpm for 10 min was conducted. Finally, the pellets were resuspended in 10 ml denature buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.9 and 6 M Urea) and incubated at 37°C for 30 min to denature the insoluble fusion proteins. The fusion proteins were further isolated by slab-gel electrophoresis method (Yeh and Gonsalves, 1984). Proteins were eluted from polyacrylamide gel by Electro-Eluter Model 442 (Bio-RAD, Hercules, CA). Yields of the purified proteins were...
estimated by comparison with bovine serum albumin (BSA) in 12% SDS-PAGE using the Spot Density software of AlphaInnotech IS2000 (AlphaInnotech Corporation, San Leandro, CA) as previously described (7).

Production of mouse monoclonal antibodies (MAbs)

Fifty microgram of the purified pET-expressed CCYV CP solved in 250 μl PBS was emulsified with an equal volume of Freund’s complete adjuvant (Difco Laboratories, BD, Franklin Lakes, NJ) and injected intraperitoneally into 6- to 8-week-old female BALB/cByJ mice (Academic Sinica, Taipei, Taiwan). One week later, 50 μg of the same immunogen in 250 μl PBS was emulsified with an equal volume of Freund’s incomplete adjuvant (Difco Laboratories) and administered twice at weekly intervals. Mice were sacrificed 3 days after a final injection with 50 μg immunogen in 250 μl PBS without adjuvant at the fourth week, and their splenocytes were harvested for cell fusion with Sp2/0-Ag14 myeloma cells provided by Dr. Wan (China Medical University Hospital, Taichung, Taiwan) following a previously described method with modifications (24). After fusion, hybridoma cells were incubated at 37°C supplied with 6% CO₂.

Cultured media were collected and used to screen desired antibodies by both indirect enzyme-linked immunosorbent assay (ELISA) and western blotting. Subsequently, the antibody-secreting hybridoma cells were cloned by limiting dilution. Stable hybridoma cell lines were selected by three cycles of cloning and then injected intraperitoneally into the Pristane-primed BALB/cByJ mice with 1.0 × 10⁶ cells for production of ascitic fluids.

Indirect ELISA

Indirect ELISA was carried out according to the method described previously with modifications (65) for screening the desired antibodies. Each well of polystyrene microtitration plates was coated with 10 ng of the pET-expressed fusion CCYV CP in coating buffer (15 mM Na₂CO₃, 34 mM NaHCO₃ and 3 mM NaN₃). The plates were incubated at 37°C for 1 hr, and then washed with PBST buffer (136 mM NaCl, 1 mM KH₂PO₄, 8 mM Na₂HPO₄·12 H₂O, 2 mM KCl, 3 mM NaN₃ and 0.05% Tween 20) for three times (each time for 3 min). The cultured media used as primary antibodies were loaded in the pre-coated plates. The plates were incubated at 37°C for 1 hr and then washed three times with PBST buffer. The alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody at a 1:5000 dilution in conjugate buffer (PBST containing 2% PVP-40 and 0.2% ovalbumin). The plates were incubated at 37°C for 1 hr and then washed with PBST buffer for three times. Color-developing solution prepared by dissolving ρ-nitrophenyl phosphate disodium hexahydrate (ρ-NPP) in substrate buffer (9.7% diethanolamine containing 3 mM NaN₃) to a final concentration of 1 mg/ml, were added for colorization. Readings of the absorbance at 405 nm (A₄₀₅) were recorded by a Victor³ 1420 (PerkinElmer, Multilable Counter, Waltham, MA) at 30 to 60 min after the addition of enzyme substrate.

Western blotting

For MAb screening and field virus detection, the western blotting was employed as described previously (20) with modifications. Leaves of the virus-infected plants were ground at a 1:5 dilution in protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 12% glycerol, 0.01% bromophenol blue and 2% β-mercaptoethanol). The extracts were boiled for 3 min and the lysates were removed by centrifugation at 13,300 rpm for 3 min. The supernatants were separated in 15% SDS-PAGE and then transferred onto nitrocellulose (NC) membranes in transfer buffer (25 mM Tris-HCl, pH 7.4, 192 mM glycine and 20% methanol) using a Bio-Rad mini transblot cell at 120 V for 30 min. The NC membranes were washed and blocked with TSW buffer (10 mM Tris-HCl, pH 7.4, 154 mM NaCl, 4% gelatin, 0.1% Triton X-100 and 2% SDS) for 3 times (each time for 3 min) and then incubated with TSW buffer-diluted polyclonal or monoclonal antibodies for 1 hr. RAs-His was used at 1:2000 dilution for detection the
histidine-tagged protein. The produced mouse antiserum (MAs) and MAbs were diluted at proper dilutions for use. The AP-conjugated goat anti-rabbit and mouse IgGs (Jackson) were used to react with RAs-His and mouse MAbs, respectively, at a 1:5000 dilution at 25℃ for 1 hr. The NC membranes were then washed twice with TSW buffer and rinsed with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM MgCl₂) for 3 min. Color development was conducted by adding 50 μl of NBT (nitro blue tetrazolium chloride) and 25 μl of BCIP (5-bromo-4-chloro-3-indoyl phosphate) in 7.5 ml substrate buffer. Reactions were stopped by submerging NC membranes in water.

### Tissue blot immunoassay (TBIA)

TBIA (22) was performed with modifications from the procedures of western blotting. The transverse sections of the petioles of melon plants were imprinted on NC membranes within the aforementioned transfer buffer of western blotting for 30 min. The NC membranes were washed with TSW buffer for 3 times (each time for 3 min) and then incubated with TSW buffer-diluted MAb to the CCYV CP or the MYSV NP at a 1:500 dilution at 25℃ for 1 hr. After 3 washes in TSW, the AP-conjugated goat anti-mouse IgG (Jackson) was used at a 1:5000 dilution to react with mouse MAbs at 25℃ for 1 hr. The NC membranes were then washed twice with TSW buffer and rinsed with substrate buffer for 3 min. Color development was conducted by adding 50 μl of NBT and 25 μl of BCIP in 7.5 ml substrate buffer. Reactions were stopped by submerging NC membranes in water.

### Identification of Crinivirus-TW as an isolate of CCYV

Ten overlapping RT-PCR-amplified DNA fragments from the Crinivirus-TW-infected N. benthamiana plant were cloned and sequenced. The complete nt sequence of the RNA2 assembled from these ten contigs is determined as 8,041 nt in length (GenBank accession number JF502222) to encode eight ORFs, including P5, Hsp70h, P6, P60, P9, CP, CPm and P26. The virus isolate was further verified as CCYV, denoted CCYV-TW, based on the RNA2 sequence shares 99% nt identity with that of the original Japanese isolate of CCYV (designated as CCYV-JP in this study). The hallmark genes of Closteroviridae were analyzed. The Hsp70h coding region of CCYV-TW has 1,671 nt, starting from the nt 1207 and terminating at the nt 2,877, to encode a 556 aa (62.5 kDa) protein. The P60 coding region of CCYV-TW is 1,554 nt in length, ranging from the nt 3,036 to the nt 4,589, encoding a protein of 517 aa (59.9 kDa). The CP coding region of CCYV-TW has 753 nt, ranging from the nt 4,941 to the nt 5,693, encoding a protein of 250 aa (28.7 kDa). The CPm coding region of CCYV-TW has 1,425 nt, ranging from the nt 5,693 to the nt 7,117 to encode a 474 aa (54.5 kDa) protein. The P26 coding region of
Fig. 1. Symptoms on a field melon (Cucumis melo L.) plant caused by the new emerging Crinivirus-like virus. Symptoms of chlorotic spots on the upper leaf (A), and bleaching accompanied with green veins and brittleness on the lower leaf (B) of a diseased melon plant. (C) The presence of the new emerging crinivirus in the diseased melon plants and the silverleaf whiteflies (Bemisia argentifolii Bellows & Perring), both collected from field, were verified by reverse transcription-polymerase chain reaction using the primers Crini-hsp70-f and Crini-hsp70-r. Total RNA extracted from a healthy melon plant (H) and a virus-free silverleaf whitefly (vf) were used as the negative controls. The size of amplicons is indicated by an arrow.

Fig. 2. Symptom development on a Nicotiana benthamiana plant inoculated with a Taiwan isolate of crinivirus (denoted as Crinivirus-TW) by silverleaf whitefly (Bemisia argentifolii Bellows & Perring) transmission. Symptoms showing yellow and necrotic spots on the lower leaf (A) and chlorotic spots on the upper leaf (B) of a N. benthamiana plant were observed at 30 days post-inoculation. (C) The presences of Crinivirus-TW in the symptomatic N. benthamiana plant and the virus-acquired silverleaf whitefly individuals were confirmed by reverse transcription-polymerase chain reaction using primers Crini-hsp70-f and Crini-hsp70-r. Total RNA extracted from leaves of a healthy N. benthamiana plant (H) and a virus-free silverleaf whitefly (vf) were used as the negative controls. The size of amplicons is indicated by an arrow.
CCYV-TW has 642 nt, ranging from the nt 7,179 to the nt 7,820 to encode a 213 aa (25 kDa) protein. The Hsp70h, P60, CP, CPm and P26 of CCYV-TW are closely related to those of CCYV-JP sharing 99.5-100% of aa identity (Table 3). Moreover, based on the Hsp70h, P60, CP, CPm and P26, phylogenetic analyses revealed that CCYV, LCV, BYDV and CYSDV are closely related to each other (Fig. 3).

Table 3. Nucleotide (nt) and amino acid (aa) identity (%) of the coding regions of CCYV-TW compared with those of the Japanese isolate CCYV-JP and other criniviruses

For virus abbreviations, see Table 1.

<table>
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<tr>
<th>Species</th>
<th>Hsp70h</th>
<th>P60</th>
<th>CP</th>
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<td>nt</td>
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<tr>
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**Production of MAbs against the CP of CCYV-TW**

An estimated 31 kDa of recombinant CCYV-TW CP with a histidine tag at the N-terminal end was expressed and confirmed by western blotting using RAs-His. The 400 µg expressed CP was purified from a 320 ml-bacterial culture and used as an immunogen for preparation of MAbs. The stable hybridoma cell lines, 7A11F8, 11G7D12 and 16C10A7, secreting antibodies against the immunogen were confirmed by indirect ELISA. Antibodies produced by all hybridoma cell lines were classified as the IgG1 isotype with Kappa light chains. Ascitic fluids stimulated by these hybridoma cells produced from the Pristane-primed mice were used at a 1/2000 dilution in our further investigation.

Mouse antiserum (denoted MAs-CCYV CP) and antibodies (denoted MAb-CCYV CP) derived from 7A11F8, 11G7D12 and 16C10A7 were able to react with the pET-expressed recombinant protein and crude extracts of CCYV-infected *Nicotiana benthamiana* plants in western blotting (Fig. 4A-D). No serological reactions were found when MAs and all MAbs incubated with the crude sap of healthy *N. benthamiana* plants.

Moreover, MAb-CCYV CP confirmed to be used for CCYV detection in various naturally infected cucurbit crops, such as melon, watermelon and cucumber (Fig. 4E). A major protein of 29 kDa and a minor band lower than 26 kDa were only observed in the CCYV-infected samples. It is considered resulting from the degradation of CP. No serological responses were observed when MAb-CCYV CP reacted with the diseased plant samples infected with MYSV, WSMoV, CMV or ZYMV.
Fig. 3. Phylogenetic relationships of the major RNA2-encoded proteins of CCYV-TW with those of other criniviruses. The Hsp70h (A), P60 (B), CP (C), CPm (D) and P26 (E) were used for comparison. The dendrographs were produced using the Neighbour-Joining algorithm with 1000 bootstrap replicates. Virus abbreviations are listed in Table 1. The original Japan isolate of CCYV is designated as CCYV-JP.

The presence of CCYV in phloem cells

CCYV was detected in the phloem cells of the infected melon plants by TBIA using MAb-CCYV CP (Fig. 5A). No serological reactions were found in cells of the healthy melon plants (Fig. 5B). Additionally, no reactions were observed when MAb-MYSV NP (9) was used to incubate with the samples of both CCYV-infected and healthy melon plants (Fig. 5C and D). Results indicated that the serological reaction is specific and CCYV is phloem-limited.

Field survey for melon plants infected with CCYV in central Taiwan

Primer pairs Crini-hsp70-f/Cirni-hsp70-r (29) and CCYV-HSP-f/CCYV-HSP-R (21) were used to amplify 0.39 bp and 0.46 bp of DNA fragments, respectively, from the CCYV-infected samples (Fig. 6). No PCR fragments were amplified from the diseased plant samples infected with MYSV, WSMoV, CMV or ZYMV. The fragment amplified by Crini-hsp70-f/Cirni-hsp70-r from all
Fig. 4. Serological reactions of mouse antiserum (MAs-CCYV CP) and monoclonal antibodies (MAb-CCYV CP) against the coat protein (CP) of CCYV-TW in western blotting. The MAs-CCYV CP (A) and the ascitic fluids produced from the hybridoma cell lines 7A11F8 (B), 11G7D12 (C) and 16C10A7 (D) were used at a 1:2000 dilution to react with crude extracts of CCYV-infected *Nicotiana benthamiana* plants (lane 1). The crude sap extracted from a healthy *N. benthamiana* plant (H) was used as a negative control (lane 2). Thirty nanogram of the pET-expressed CCYV-TW CP was used as a positive control (lane 3). (E) Diagnosis of CCYV infection in the natural cucurbit crops. The diseased samples of melon (M), watermelon (Wm) and cucumber (Cu) were collected from fields. The crude sap extracted from a healthy melon plant was used as the negative control (H). The crude sap extracted from leaves of *Nicotiana benthamiana* plants infected with WSMoV, MYSV and CMV, and those of *Chenopodium quinoa* infected with ZYMV were also used as negative controls. The CP of CCYV is indicated by an arrow.
CCYV-infected cucurbit samples are stronger than those amplified by CCYV-HSP-f/CCYV-HSP-R. The primer pairs Crini-hsp70-f/Crini-hsp70-r and mt-F2/mt-R1 are recommended to be used in multiplex one-step RT-PCR for detecting CCYV in field.

A total number of 253 symptomatic melon samples were collected from planted fields in Yunlin County during a cultivated season from February to June in 2010. In February, 20 of 52 melon samples (3.8%) infected with CCYV were detected. In March, 17 of 75 melon samples (22.7%) infected with CCYV were found. In April, 39 of 61 melon samples (63.9%) infected with CCYV were found. In May, 26 of 35 melon samples (74.3%) infected with CCYV were found. In June, all 45 melon samples (100%) infected with CCYV were found. The increased detection ratios of 3.8% to 100% were noticed.

Discussion

CCYV, a new species of the genus *Crinivirus* (47), causing yellows and chlorosis with brittleness on leaves of melon plants was first discovered in Taiwan in the fields of Lunbei Township, Yunlin County (29). According to the previous studies, *Bemisia tabaci* Q- and B-biotypes are the
transmission vectors of CCYV \(^{(21)}\). \(B. \) tabaci B-biotype is also known as silverleaf whitefly \((B. \) argentifolii\) which is common in melon fields. Our results provided direct evidence that silverleaf whitefly in Taiwan can transmit CCYV resulting from the virus can be directly detected on whitefly individuals and the virus can be transmitted to \(Nicotiana \) benthamiana by whitefly acquisition experiment. In Taiwan, silverleaf whitefly is also the transmission vector of \(SqLCPV\) \(^{(40)}\) which is a species of the genus \(Begomovirus\) causing chlorosis and leaf curl on melon plants at the early growing stage. The infections of CCYV and \(SqLCPV\) could be easily identified by symptomatology.

\(Crinivirus\) is phloem-limited and can not be mechanically transmitted. Thus, it is difficult to isolate virus from the infected plants \(^{(11)}\). To solve this problem, virus-free silverleaf whiteflies were used to acquire the virus from the diseased melon plants collected from field and maintained on \(N. \) benthamiana plant. In our study, symptoms of chlorotic to necrotic spots observed on the lower leaves of the inoculated \(N. \) benthamiana plants are different from those described previously \(^{(47)}\). Based on the sequence analyses of the RNA2, the virus isolate was further identified as an isolate of CCYV, denoted as CCYV-TW, which is not divergent from the original Japan isolate CCYV-JP \(^{(47)}\). CCYV was first discovered in Japan in 2004 and also found in China. However, it was found in Taiwan in 2009. It demonstrates that this virus may be imported to Taiwan from Japan or China. On the other hand, phylogenetic analyses indicated that CCYV is closely related to \(LCV\), \(BYDV\) and \(CYSDV\). The serological relationship of the four viruses needs to be further investigated.

To understand the incidence of cucurbits infected with CCYV in Taiwan, it is important to develop an effective diagnostic method for the virus detection. Several primer pairs were tested in various cucurbit crops. The primer pair Crini-hsp70h-f/Crini-hsp70r designed from the Hsp70h gene of CCYV-TW \(^{(29)}\) coupled with the primer pair mt-F2/mtR1 designed from the plant mitochondrial NADH
dehydrogenase (nad5) gene (39) are recommended for use in multiplex one-step RT-PCR for detecting CCYV in field survey.

Additionally, the CP of CCYV-TW was expressed for production of MAbs. MAbs to the CCYV-TW CP were successfully used to detect CCYV in western blotting, but not in ELISA resulting from high background readings in the healthy controls. This may be caused by the MAb cross reaction with plant components. TBIA assays indicated that CCYV accumulates in the phloem cells only, that is different from other plant viruses, such as tospoviruses. However, in western blot analyses, a major protein of 29 kDa and a minor band lower than 26 kDa were only observed in the CCYV-infected samples. It is considered resulting from the degradation of CP.

Field survey for CCYV infecting melon plants in central Taiwan during a cultivated season from February to June in 2010 was conducted. The detection ratio of CCYV increased and reached to 100% at the late cultivated stage was noticed. The incidence of CCYV in melon plants was lower at the first two months of a cultivated season, suggesting that prevention of CCYV infection needs to be taken at the nursery stage. Additionally, CCYV not only infected melon plants but also infected other cucurbits such as watermelon, cucumber, bottle gourd, squash and bottle gourd in Taiwan (29). CCYV has become an important threat for the production of cucurbits in Taiwan and should be deeply concerned by the authorities. The developed diagnostic techniques used for field survey provide valuable information and also useful for the establishment of a healthy germplasm scheme of cucurbits to control of CCYV.

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Taiwan New Melon褪绿黄化病毒分子特性与血清学诊断之研究

Plant Dis. 76: 835-840.
摘 要

李如婷、黃莉欣、曾獻嫺、高衛婷、陳宗祺。2013。台灣新興瓜類褪綠黃化病毒分子特性與血清學診斷之研究。植物病理會刊 22：259-278。

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**Crinivirus** 為 **Closteroviridae** 科之一病毒屬，為長絲狀病毒，具有由兩條正極性單股 RNA 所組成之基因體，分別命名為 RNA1 及 RNA2，且其病毒顆粒較短，介於 650-900 nm × 12 nm 之間，是由粉蝨以半永續性的方程式傳播。Crinivirus 危害全球許多重要的經濟作物，造成植株的葉片呈現黃色斑點、褪綠黃化、甚至白化的病徵，導致收成量降低而造成嚴重的經濟損失。在 2009 年四月間，於台灣雲林縣崙背鄉的洋香瓜田中，發現洋香瓜上出現典型的 Crinivirus 病徵。採集洋香瓜的罹病組織並以針對 Closterovirus 及 Crinivirus 所設計之簡併性引子對進行巢式反轉錄-聚合酶鎖反應 (nested reverse transcription-polymerase chain reaction, nested RT-PCR)，可增幅出 0.5 kb 的 DNA 片段，經定序及比對分析得知與日本所發現的新興瓜類褪綠黃化病毒 (Cucurbit chlorotic yellows virus, CCYV) 有 100%的相同度。本研究以粉蝨傳毒方式將新興病毒感染於菸草 (Nicotiana benthamiana) 中，以獲得一 crinivirus 分離株，命名為 Crinivirus-TW。將 Crinivirus-TW 的 RNA2 完全解序，可得其全長度有 8,041 個核苷酸，可對應產生八個蛋白，分別為 P5、Hsp70h、P6、P60、P9、CP、CPm 和 P26。RNA2 的核苷酸序列與日本原始的 CCYV 分離株序列具有 99%的相同度，因此 Crinivirus-TW 應為 CCYV 的分離株，並更名為 CCYV-TW。將所有已知 Crinivirus 病毒種的 Hsp70h、P60、CP、CPm 及 P26 等基因進行親緣關係比對，結果得知 CCYV-TW 與 Lettuce chlorosis virus (LCV)、Bean yellow disorder virus (BYDV) 及 Cucurbit yellow stunting disorder virus (CYSDV) 最為親近。利用 pET 細菌表現系統表現 CCYV-TW 的 CP 用以生產單株抗體 (monoclonal antibody)，所得之單株抗體可用於西方墨點法 (western blotting) 以進行田間之病毒檢測，並可應用於組織點浸免疫分析法 (tissue blot immunoassay, TBIA) 以檢視病毒於韌皮部細胞累積之情形。此外，設計自 Hsp70h 序列的引子對 Crini-hsp70h-f Crini-hsp70r 與設計自植物粒線體中的 NADH dehydrogenase (nad5) 基因之引子對 mt-F2/mtR1 共同使用所開發的 multiplex one-step RT-PCR，為相當有效率的檢測方法，可應用於田間 CCYV 之檢測。以此方法進行田間調查，2010 年 2-6 月間，自台灣中部地區共採集 253 個罹病洋香瓜樣本，檢測結果發現，病毒之檢出率從 3.8%上升至 100%。本調查結果顯示 CCYV 已成為台灣瓜類作物生產上的一大重要限制因子，值得密切注意。

關鍵詞：**Crinivirus** 屬、瓜類褪綠黃化病毒、洋香瓜