Identification of Capsicum Chlorosis Virus Causing Chlorotic Spots and Stripes on Calla Lily

Tsung-Chi Chen, Chin-An Chang, Ya-Chi Kang, Shyi-Dong Yeh, Chun-Huei Huang, and Chin-Chih Chen

Abstract


Calla lily (Zantedeschia spp.) is one of the economically important ornamental crops in Taiwan. In a field survey of calla lily conducted during 2005, plants showing symptoms of yellow spots and stripes on leaves were observed in Houli Township, one of the major areas for commercial production of ornamental crops in Taiwan. Fifteen virus isolates were collected from diseased plants of calla lily and purified via three successive local-lesion isolations on leaves of inoculated Chenopodium quinoa. A 0.9 kb DNA fragment was amplified from total RNA extracted from all the fifteen virus isolates on infected plants by reverse transcription-polymerase chain reaction (RT-PCR) using the Tospovirus genus-degenerate primers gL3637 and gL4510c, designed from the conserved regions of L RNA, revealing that the disease was caused by a Tospovirus. The virus isolates reacted positively with the antisera to the nucleocapsid (N) protein of Capsicum chlorosis virus (CaCV) and the monoclonal antibody to the N protein of Watermelon silver mottle virus (WSMoV), indicating that they are members of the WSMoV serogroup. The nucleotide sequences of the N gene of these virus isolates from calla lily were phylogenetically related to CaCV. Furthermore, the pathogenicity of CaCV was also verified by inoculation tests on plants of calla lily.

Key words: Virus disease of calla lily, Tospovirus, Capsicum chlorosis virus (CaCV).

Introduction

Calla lily (Zantedeschia spp.), belonging to Araceae, is a popular ornamental crop in Taiwan and many other countries. Nine viruses have been reported as causal agents of calla lily in Taiwan, including Calla lily latent virus (CLLV) (Chen et al. 2006b, 2006c), Dasheen mosaic virus (DsMV) (Zettler & Hartman 1987, 1995), Konjak mosaic vi-
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The genus *Tospovirus*, transmitted by thrips in a persistent manner, is the only plant-infecting genus in the family *Bunyaviridae* (Fauquet et al. 2005). The enveloped quasi-spherical particles of tospoviruses are 80 to 110 nm in diameter and contain a tripartite single-stranded (ss) RNA genome denoted large (L), middle (M) and small (S) segments (Fauquet et al. 2005). The negative sense L RNA encodes a large RNA-dependent RNA polymerase in the viral complementary (vc) strand for virus replication (de Haan et al. 1990). Both M- and S-RNAs are ambisense and each contains two open reading frames (ORFs). The viral (v) strand of M-RNA encodes a nonstructural (NSm) protein for cell-to-cell movement of non-enveloped ribonucleocapsid structures (Kormelink et al. 1994; Lewandowski & Adkins 2005), while its vc strand encodes the precursor of Gn and Gc glycoproteins for composing spikes on the viral envelope (Kormelink et al. 1992; Law et al. 1992). Another nonstructural (NSs) protein encoded by the v strand of S RNA is a gene-silencing suppressor responsible for counteracting the defense mechanisms in plants (Bucher et al. 2003; Takeda et al. 2002), and forms filamentous inclusion bodies in the infected cells (Kormelink et al. 1991). The vc strand of S-RNA encodes the nucleocapsid (N) protein for encapsidation of viral RNAs (de Haan et al. 1990).

A threshold of 90% amino acid identity in N protein is the key criterion for demarcation of tospoviruses at species level (Goldbach & Kuo 1996). In addition, tospoviruses can be clustered in serogroups or classified as serotypes on the basis of the serological and phylogenetic relationships of N proteins (Adam et al. 1993). The serological-related viruses are grouped as a serogroup, and viruses without serological relationship are designated as monospecies serotypes. So far, there are 22 formal and tentative tospovirus species that have been characterized (Seepeiban et al. 2011; Zhou et al. 2011). Most of them were claded into three major serogroups designating from type members, *Tomato spotted wilt virus* (TSWV), *Watermelon silver mottle virus* (WSMoV) and *Iris yellow spot virus* (IYSV). Three tospovirus species, *Impatiens necrotic spot virus* (INSV), *Peanut yellow spot virus* (PYSV) and *Peanut chlorotic fan-spot virus* (PCFV), were classified as monospecies serotypes (Chen et al. 2010).

Capsicum chlorosis virus (CaCV), a member of WSMoV serogroup, was first found to infect capucicum and tomato in Queensland, Australia (McMichael et al. 2002) and further recognized as a widespread pathogen in solanaceous crops in Thailand (Chiemsupradit et al. 2008; Knierim et al. 2006). Recently, it has become an important quarantine virus for the production of *Phalaenopsis* orchids in Taiwan (Zheng et al. 2008). In 2005, symptoms of yellow spots and stripes on leaves of calla lily plants were found in cultivated fields in Houli Township, Taiwan. Fifteen virus isolates were collected from diseased plants by single lesion isolations. All fifteen virus isolates were further identified as isolates of CaCV by sequence determination of their N genes (Chen et al. 2007a). In this study, one of calla lily isolates of CaCV (FG1) was back inoculated to calla lily plants to verify that CaCV is the natural causal agent of chlorotic spots and stripes of calla lily. The molecular relationships of the calla lily isolates of CaCV were also analyzed.

**Materials and Methods**

**Virus inoculation**

Fifteen isolates of CaCV collected from calla lily in Houli Township, Taichung, Taiwan in 2005 were previously described by Chen et al. (2007a). Other viruses used in this study were: one isolate of CaCV from gloxinia, denoted HT-1, which was collected in the United States by Dr. H.-T. Hsu (Hsu et al. 2000) and one isolate of WSMoV from watermelon (Yeh et al. 1992) and one isolate of CCSV from calla lily (Chen et al. 2005) in Taiwan. All tospoviruses were maintained on leaves of *Chenopodium quinoa* and *Nicotiana benthamiana* by mechanical inoculation. A calla lily isolate of CaCV, denoted FG1, was mechanically inoculated on leaves of calla lily seedlings (*Zantedeschia eliottiana* cv. ‘Pot of...
The virus-infected tissues were ground with 0.05 M potassium phosphate buffer (pH 7.5) in a ratio of 1:10 (w/v) and the crude saps were introduced onto leaves of calla lily plants. The inoculated plants were kept in a screenhouse used for further serological and molecular assays.

**Indirect enzyme-linked immunosorbent assay (ELISA)**

The procedures of indirect ELISA were conducted as previously described (Chen et al. 2003; Clark & Adams 1977). The rabbit antisera to the calla lily-infecting viruses were prepared in our laboratory and used for serological tests of viruses, including CarMV, CMV, CLLV, DsMV, KoMV, TuMV and ZaMMV (Chen et al. 2006a, 2006c; Chen et al. 2007a), the antiserum against the N protein of CaCV HT-1 (Hsu et al. 2000), the monoclonal antibodies (MAbs) against the N protein of CCSV and WSMoV (Lin et al. 2005) and the Potyvirus-general MAb (Agdia, Elkhart, IN). The alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG and the AP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a 1:5000 dilution as the secondary antibodies for detecting rabbit and mouse antibodies, respectively. Absorbance at 405 nm (A$_{405}$) was recorded by PTI max microplate reader (Molecular Devices, Sunnyvale, CA) 30 min after the addition of r-nitrophenyl phosphate substrate (1 mg/mL) (Amresco, Solon, OH).

**Cloning and sequencing**

Extraction of total RNAs of virus-infected plant tissues using the Plant Total RNA Miniprep Purification Kit (Hopegen, Taichung, Taiwan), and reverse transcription (RT) of the first strand cDNA were conducted as described previously (Chen et al. 2006b). The primer pairs, gL3637 [5'-CCTTTAACAGT(A/T/G)GAAACAT-3']/gL4510c [5'-TCATC(A/G)GA(A/G)TG(T/G/C)AC(A/C)ATCCATCT-3'] (Chu et al. 2001), designed from the conserved regions of tospoviral L RNAs, and WN2328 (5'-CCATTG-GTTGCCCTCCG-3')/WN3534 (5'-CGTGCACA-GGCAATCGAGGC-3') (Chen et al. 2007a), designed from the S RNA of WSMoV, were used for amplification of a partial L gene fragment and a full-length N gene, respectively. Polymerase chain reaction (PCR) amplification was carried out by 26 cycles: denaturing at 94°C for 1 min, annealing at 50°C for 45 sec, and DNA synthesis at 72°C for 90 sec. An elongation step at 72°C for 6 min was conducted at the last additional cycle. Amplified DNA products were analyzed by electrophoresis in a 1% agarose gel. The amplified DNA fragments corresponding to the full-length N genes were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Nucleotide sequences were determined by an automatic DNA sequencer (ABI PRISM 377, Perkin-Elmer, Foster City, CA). Three independent clones were selected for alignment to determine the correction of nucleotide sequences.

**Sequence and phylogenetic analyses**

Prediction of open reading frames (ORFs) in the determined sequences was conducted using the ORF program of Vector NTI (Invitrogen). Multiple sequence alignments were carried out by the Clustal W program of Vector NTI. The amino acid sequence alignments were performed by the Align X program of Vector NTI. The phylogenetic relationships among different virus isolates, according to the complete amino acid sequences of N proteins, were also analyzed. The N protein sequences of WSMoV (accession no. U78734), CaCV (accession no. UY036057), CCSV (accession no. UY867502), TSWV (accession no. D13926), INSV (accession no. X66972), and PCFV (accession no. AF080526) were used for comparison. Analyzed sequences were first aligned using Clustal X version 1.8 (Jeanmougin et al. 1998). Their phylogenetic relationships were determined using PAUP 4.0 (Swofford, 1998) by the Neighbour-Joining algorithm with the bootstrap resampling method (Felsenstein 1985; Thompson et al. 1997). One thousand random resamplings were used to calculate the bootstrap values. The calculated trees were displayed by the TreeView program (Page 1996).

**Primer design for virus identification**

Specific primers, CaCV4f (5’-TCTACCGT CAGGAACCTTACCGAG-3’) and CaCV777c (5’-ATAATCATCCACAGAAATTGGCAC-3’), were designed to identify CaCV from the infected plant tissues. RT-PCR was conducted by One-Step RT-PCR Kit (Hopegen, Taichung, Taiwan) as the manufacturer’s instruction. The first strand
cDNAs were synthesized at 50°C for 30 min and terminated at 94°C for 2 min, and then PCR was performed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. The amplicons were analyzed by electrophoresis in 1% agarose gels.

**Results**

**Isolation and identification of virus from calla lily in Taiwan**

In 2005, symptoms of chlorotic spots and stripes accompanying necrosis on leaves of calla lily plants were found in cultivated fields in Houli Township, Taiwan (Fig. 1A). Fifteen symptomatic calla lily samples were collected from field and tested in indirect ELISA. All of the tested samples did not react with the *Potyvirus*-general MAb (Agdia) and the antisera to CarMV, CLLV, CMV, DsMV, KoMV, TuMV and ZaMMV, which are common calla lily-infecting viruses (Chen et al. 2003, 2006b; Huang & Chang 2005; Zettler & Hartman 1995). The *Tospovirus* genus-degenerate primers gL3637 and gL4510c (Chu et al. 2001) were used in RT-PCR to amplify a 0.9-kb DNA fragment from all samples (Fig. 2). Thus, the calla lily-infecting virus isolates were predicted as tospoviruses. Furthermore, the antiserum to CaCV HT-1 (denoted RAs-CaCV) and the MAbs to CCSV and WSMoV were used in indirect ELISA to verify these calla lily virus isolates. All samples were positively reacted with RAs-CaCV and the MAb to WSMoV (denoted MAb-WSMoV), but not reacted with MAb to CCSV (data not shown). The results demonstrated that the diseased calla lily samples collected from Houli Township were infected by a *Tospovirus* of WSMoV serogroup.

**Isolation and back-inoculation of calla lily-infecting tospoviruses**

Fifteen virus isolates designated as BM11, BM12, BM13, BM14, BM17, FG1, FG2, FG3, FG13, PG5, PG22, PG23, PG25, PG28 and PG30, were obtained from successive single-lesion isolations on inoculated chenopodium plants. All virus isolates produced similar necrotic local lesions on the inoculated leaves of *C. quinoa* plants (Fig. 1B) and they produced typical symptoms of leaf curl and mosaic on leaves of inoculated plants of *N. benthamiana* (Fig. 1C). The individual virus isolates were confirmed by positively reacting with RAs-CaCV and MAb-WSMoV in indirect ELISA (data not shown). The FG1 isolate was mechanically introduced to ten calla lily seedlings and the results showed that one of the inoculated seedlings showed yellow spots on the newly extended leaf (Fig. 1D). An average ELISA reading of 0.38, two-fold higher than the reading of negative control (0.08), was obtained when the symptomatic calla lily tissue reacted with RAs-CaCV in indirect ELISA to confirm the CaCV infection.

**Sequence and phylogenetic analyses of the N genes of the calla lily isolates of CaCV**

The primers WN2328 and WN3534, designed from the intergenic region and the 3′-untranslatable region of the S-RNA of WSMoV, respectively, were used to amplify a 1.1-kb DNA fragment from total RNA extract of the aforementioned CaCV isolates. Sequence determination revealed that the amplified DNA fragments correspond to full-length N genes. The N gene sequences of the fifteen virus isolates were submitted to GenBank to obtain accession numbers (Table 1). The N genes of all fifteen CaCV isolates share a high homology of 95.8–97.1% nucleotide sequence identities and 97.5–98.2% amino acid sequence identities with that of the typical CaCV Australian isolate (accession no. AY036057), but a lower homology of 76.7–78.1% nt identities and 84.4–86.2% aa identities with that of the original WSMoV isolate from Taiwan (accession no. U78734). Phylogenetic analysis of the N proteins of fifteen calla lily-infecting CaCV isolates and other tospoviruses revealed that these isolates of CaCV can be divided into two groups: isolates BM17, FG1, FG3, FG13, PG23, PG28 and PG30 were closely related to HT-1 and were clustered as a group, while isolates BM11, BM12, BM13, BM14, FG2, PG5, PG22 and PG25 were clustered as another group (Fig. 3).

**Identification of CaCV by RT-PCR**

A 0.77-kb DNA fragment was amplified from all tested samples of CaCV isolates by RT-PCR using the primers CaCV4f and CaCV777c, but no signals were obtained from WSMoV (Fig. 4). The results indicated that CaCV can be distinguished from WSMoV by the newly designed specific primers in RT-PCR.

**Discussion**

In addition to previous reports of *Tospovirus*, such as TSWV (Zettler & Hartman 1995) and CCSV
Fig. 1. Symptoms of calla lily-infecting virus isolates on calla lily (A, D), Chenopodium quinoa (B) and Nicotiana benthamiana (C). (A) Symptoms of yellow spots and stripes on a leaf of a calla lily plant grown in the field; (B) Local lesions produced on a leaf of Chenopodium quinoa inoculated with a calla lily-infecting virus isolate FG1; (C) Tospovirus-caused chlorotic spot symptoms were observed on the leaves of Nicotiana benthamiana inoculated with a single-lesion isolated virus isolate FG1; and (D) Yellow spots produced on a systemic leaf of calla lily inoculated with the FG1 isolate after inoculation for 21 days.

Fig. 2. Detection of fifteen calla lily-infecting virus isolates by reverse transcription-polymerase chain reaction. The degenerate primer pair gL3637/gL4510c (Chu et al. 2001), designed from the consensus sequences of tospoviral L RNAs, was used to amplify a 0.9-kb DNA fragment from total RNAs extracted from the naturally infected calla lily samples. Lane M, 100 ladder marker; lane 1 to 15, virus isolates BM11, BM12, BM13, BM14, BM17; FG1, FG2, FG3, FG13; PG5, PG22, PG23, PG25, PG28 and PG30; lane C, Capsicum chlorosis virus (CaCV) gloxinia isolate HT-1; lane W, Watermelon silver mottle virus (WSMoV); and lane H, healthy calla lily as negative control.
(Lin et al. 2005) on calla lily, this study reveals another tospovirus CaCV responsible for the disease of calla lily occurred in Houli (Taichung, Taiwan) in 2005 was verified to invade calla lily naturally. Diseased calla lily plants infected with CaCV did not mix-infected with other viruses were confirmed by serological tests using various antisera and MAbs to calla lily-infecting viruses and bioassays via single-lesion isolation. Symptoms caused by various viruses on calla lily plants in Taiwan were similar. For instance, symptoms caused by CaCV are similar to those induced by TuMV and CCSV, showing green, yellow or chlorotic spots and strips on the infected leaves of calla lily plants (Chen et al. 2003, 2005). For this reason, diagnosis of calla lily virus diseases based on symptoms alone is difficult and unreliable.

Serological and molecular analyses for identification of calla lily-infecting viruses are recommended. Genus broad-spectrum degenerate primers and antibodies are effective tools for preliminary diagnosis of virus diseases in the field. In this study, field samples of diseased calla lily were successfully identified as tospovirus infections by RT-PCR using the Tospovirus genus-degenerate primer pair gL3637/gL4510c, which was designed from the consensus sequences of tospoviral L RNAs (Chu et al. 2001). It led to a correct direction to diagnose a virus disease. CaCV was first identified in Australia as a pathogen of Capsicum spp. causing chlorotic spots on leaves (McMichael et al. 2002), and it was subsequently reported on solanaceous crops in Thailand (Chiemsombat et al. 2008) and India (Kunkalikar et al. 2010) and on peanut (Arachis hypogaea L.) in China (Chen et al. 2007b) and Thailand (Chiemsombat et al. 2008). In Taiwan, CaCV was reported as a pathogen mainly on tomato (Huang et al. 2010) and ornamental crops, such as orchids (Zheng et al. 2008), calla lily (Chen et al. 2007a), amaryllis (Hippeastrum hybridum Hort.) and blood lily (Haemanthus multiflorus Martyn.) (Chen et al. 2009).

CaCV is genomically closely related to WSMoV with homologies of 84–91% amino acid sequence identities in each viral protein (Knierim et al. 2006), and they are serologically indistinguishable.

### Table 1. Nucleotide (nt) and amino acid (aa) identities of the nucleocapsid genes of the calla lily-infecting tospovirus isolates compared with those of the typical isolates of Capsicum chlorosis virus (CaCV) and Watermelon silver mottle virus (WSMoV)

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Accession no.</th>
<th>CaCV z</th>
<th>WSMoV</th>
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<tr>
<td></td>
<td></td>
<td>nt</td>
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<tr>
<td>BM11</td>
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<td>97.5</td>
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<tr>
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<td>BM13</td>
<td>EF095727</td>
<td>97.0</td>
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<tr>
<td>BM14</td>
<td>EF095726</td>
<td>96.8</td>
<td>98.2</td>
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<tr>
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<td>PG30</td>
<td>EF100606</td>
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z Accession no. AY036057 for CaCV and accession no. U78734 for WSMoV were used for sequence comparisons.
Fig. 3. Phylogenetic analysis of the nucleocapsid (N) proteins of fifteen calla lily-infecting virus isolates and other tospoviruses. The tree was constructed using the neighbour-joining algorithm with the bootstrap resampling method (1000 random resamplings) using PAUP 4.0. Accession numbers of the N protein sequences of the individual virus isolates are listed in Table 1. The N protein sequences of the typical isolates of Capsicum chlorosis virus (CaCV; AY036057), Watermelon silver mottle virus (WSMoV; U78734), Calla lily chlorotic spot virus (CCSV; AY867502), Tomato spotted wilt virus (TSWV; D13926), Impatiens necrotic spot virus (INSV; X66972) and Peanut chlorotic fan-spot virus (PCFV; AF080526) are used for comparison.

Fig. 4. Design of specific primers for identification of Capsicum chlorosis virus (CaCV) infecting calla lily in reverse transcription-polymerase chain reaction. The primers CaCV4f and CaCV777c were designed to amplify a 0.77-kb DNA fragment from CaCV-infected samples. Lane M, 100 ladder marker; lane 1 to 15, virus isolates BM11, BM12, BM13, BM14, BM17, FG1, FG2, FG3, FG13, PG5, PG22, PG23, PG25, PG28 and PG30; lane C, CaCV gloxinia isolate HT-1; lane W, Watermelon silver mottle virus (WSMoV); and lane H, healthy calla lily.
able on the basis of N and NSs proteins (Chen et al. 2010). Our results also revealed that CaCV and WSMoV are indistinguishable by RT-PCR analysis using the WSMoV N gene primers WN2328 and WN3534, which were designed for specifically for detection of WSMoV in field survey (Chen et al. 2007a). Previous reports also indicate that CaCV, WSMoV, PBNV and WBNV are the four closely related WSMoV-serogroup tospoviruses and are difficult to distinguish by serological assays (Chen et al. 2005; Jain et al. 2007; Zheng et al. 2008). Thus, determination of the full-length N gene sequences is necessary for verifying these tospoviruses.

Development of highly specific primers for RT-PCR amplification is important for detection and identification of viruses. A previously designed primer pair WN2963/WN3469c was reported to identify WSMoV from field samples (Chen et al. 2010). Here, a CaCV-specific primer pair CaCV4f/CaCV777c was designed and successfully used in RT-PCR to differentiate CaCV from WSMoV (Fig. 4). These two primer pairs are useful and convenient tools to detect and differentiate CaCV and WSMoV in the fields. In addition to further differentiate the four serologically close-related tospoviruses, such as WSMoV, CaCV, PBNV and WBNV, a convenient one tube-based multiplex RT-PCR method using the combination of individual virus-specific primer pairs will be developed.

No insect vectors were found in the diseased calla lily fields. To our knowledge, WSMoV can be transmitted by Thrips palmi, which is a common thrips in Taiwan (Chen et al. 1990). However, the reported vector of CaCV, such as Ceratothripoides claratris (Premachandra et al. 2005), was not found in Taiwan. The category of thrips transmitting CaCV in field should be investigated.

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Capsicum Chlorosis Virus 引起彩色海芋黃化斑點及條斑之鑑定

陳宗祺 < 張清安 < 康雅琪 < 葉錫東 < 黃春惠 < 陳金枝，2012。Capsicum chlorosis virus 引起彩色海芋黃化斑點及條斑之鑑定。台灣農業研究 61: 64–74。

彩色海芋 (Zantedeschia spp.) 是國內重要之經濟花卉，本研究於2005年在后里地區觀察到彩色海芋植株葉片出現黃色斑點及條斑病徵。取不同罹病植株病葉經由連續三次單斑接種於奎藜 (Chenopodium quinoa) 後，共獲得15個純系之病毒分離株。因等病毒分離株之核酸以對應 Tospovirus L基因之廣效性引子對gL3637/gL4435c進行反轉錄-聚合酶鍵鎖反應 (Reverse-transcription polymerase chain reaction, RT-PCR)，均可獲得與預估相符約0.9 kb之DNA片段，顯示分離株均為 Tospovirus屬病毒之成員。而分離株所產生之單斑均與對應核酸蛋白 (nucleocapsid protein, NP) 之抗血清包括番茄黃化病毒 (Capsicum chlorosis virus, CaCV) 多元抗體及西瓜銀斑病毒 (Watermelon silver mottle virus, WSMoV) 單株抗體產生正反應，顯示其為 Tospovirus屬WSMoV血清群之成員。

經全長核酸蛋白基因序列及類緣演化分析結果，顯示此等病毒均為CaCV的分離株。進一步完成彩色海芋寄主之回接試驗，病毒分離株可於植株葉片造成與田間觀察到之類似的黃斑病徵，證實 CaCV為引起此病害之病毒。

關鍵詞：彩色海芋病毒病、Tospovirus屬病毒、番茄黃化病毒。

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