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FULL RESEARCH PAPER

Identification and characterization of a tospovirus causing chlorotic ringspots on *Phalaenopsis* orchids

You-Xiu Zheng · Ching-Chung Chen · Chia-Jin Yang · Shyi-Dong Yeh · Fuh-Jyh Jan

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Abstract A putative virus-induced disease showing chlorotic ringspots on leaves of *Phalaenopsis* orchids has been observed in Taiwan for several years. A virus culture, 91-orchid-1, isolated from a *Phalaenopsis* orchid bearing chlorotic ringspot symptoms was established in *Chenopodium quinoa* and *Nicotiana benthamiana*, and characterized serologically and biologically. The virus reacted slightly with the antiserum of *Watermelon silver mottle virus* (WSMoV) but not with those of *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Groundnut ringspot virus* (GRSV). Isometric particles measuring about 70–100 nm were observed. Inoculation with isolated virus was conducted to confirm that 91-orchid-1 is the causal agent of chlorotic ringspot disease of *Phalaenopsis* orchids. To determine the taxonomic relationships of the virus, the conserved region of L RNA and the complete nucleocapsid gene (N gene) were cloned and sequenced. The sequence of conserved region of L RNA shares 83.8, 82.5, 64.4 and 64.9% nucleotide identities and 96.5, 97.7, 67.3 and 67.6% amino acid identities with those of Peanut bud necrosis virus (PBNV), WSMoV, TSWV and INSV, respectively, indicating that 91-orchid-1 is a tospovirus related to WSMoV. The complete nucleotide sequence of the N gene determined from a cDNA clone was found to be 828 nucleotides long encoding 275 amino acids. Sequence analyses of the N gene showed that 91-orchid-1 is an isolate of Capsicum chlorosis virus (CaCV) which has been reported to infect tomato and capsicum plants in Australia and Thailand. 91-orchid-1 is therefore designated as CaCV-Ph. To our knowledge, this is the first formal report of a tospovirus infecting *Phalaenopsis* orchids.

Keywords Bunyaviridae · New host record · Orchid virus · Phylogeny

Y.-X. Zheng · C.-J. Yang · S.-D. Yeh · F.-J. Jan (✉)
Department of Plant Pathology,
National Chung Hsing University,
Taichung 402, Taiwan
e-mail: fjjan@nchu.edu.tw

C.-C. Chen
Taichung District Agriculture Improvement Station,
Changhua 515, Taiwan

Introduction

Phalaenopsis orchids are popular ornamental plants because of their beautiful appearance, long-lasting flowers and enormous diversity. They are one of the most important orchids grown in Taiwan for export, with approximately 150 ha of farm lands under cultivation.

More than 28 orchid-infecting viruses have been listed (Zettler et al. 1990; Lawson and Hsu 1995), and among them, *Cymbidium mosaic virus* (CymMV, *Potexvirus*), *Odontoglossum ringspot virus* (ORSV, *Tobamovirus*), *Cucumber mosaic virus* (CMV, *Cucumovirus*; Lawson and Hsu 1995), *Orchid fleck virus* (OFV, *Rhabdovirus*;

Lawson and Brannigan 1986), an uncharacterized closterovirus (Lesemann 1977) and a rhabdovirus (Lawson and Brannigan 1986) were reported to infect *Phalaenopsis* orchids. CymMV, ORSV, and CMV are the only three viruses that have been reported to infect *Phalaenopsis* orchids in Taiwan (Ko 1988; Ko et al. 1988).

Phalaenopsis plants bearing virus-like symptoms of chlorotic ringspots have been observed in central and southern Taiwan for more than a decade (Chen et al. 1998). A tospovirus-like culture was once isolated from diseased orchids (Chen et al. 1998). However, back-inoculation of this virus culture onto *Phalaenopsis* orchid to confirm the culture as the causal agent was not successful and the sequence of the nucleocapsid (N) gene for virus taxonomy was not determined at that time. As the causal agent of this orchid disease was unclear, the virus was described as 'Taiwan virus' since these symptoms were only observed on orchids from Taiwan. We report here the isolation, serological and molecular characterization, and back-inoculation of the virus causing chlorotic ringspots on *Phalaenopsis* orchids in Taiwan.

Materials and methods

Virus sources and maintenance

In 2002, a virus culture (91-orchid-1) was collected from a diseased *Phalaenopsis* orchid showing chlorotic ringspot symptoms on leaves (Fig. 1a) in central Taiwan. TSWV isolated from tomato in New York (TSWV-NY) was provided by R. Provvidenti, New York State Agricultural Experiment Station (NYSAES), Geneva, NY, USA. A Brazilian isolate of *Groundnut ringspot virus* (GRSV-BR) was provided by D. Gonsalves (NYSAES) (Pang et al. 1993). INSV collected from impatiens in the United States (INSV-M) was provided by J. Moyer, North Carolina University, Raleigh, NC, USA (Law and Moyer 1990). The isolates of *Watermelon silver mottle virus* (WSMoV) and Peanut chlorotic fan-spot virus (PCFV) used for comparison were collected from watermelon (Yeh et al. 1992) and peanut (Chen and Chiu 1996) in Taiwan. All viruses were maintained in a systemic host, *Nicotiana benthamiana*, and a local lesion host, *Chenopodium quinoa*, by mechanical inoculation using 30-fold dilution of the inoculum prepared by grinding infected leaf tissue in buffer 4 (0.033 M KH_2PO_4, 0.067 M K_2HPO_4 and 0.01 M Na_2SO_3, pH 7.0) (Jan et al. 2000).

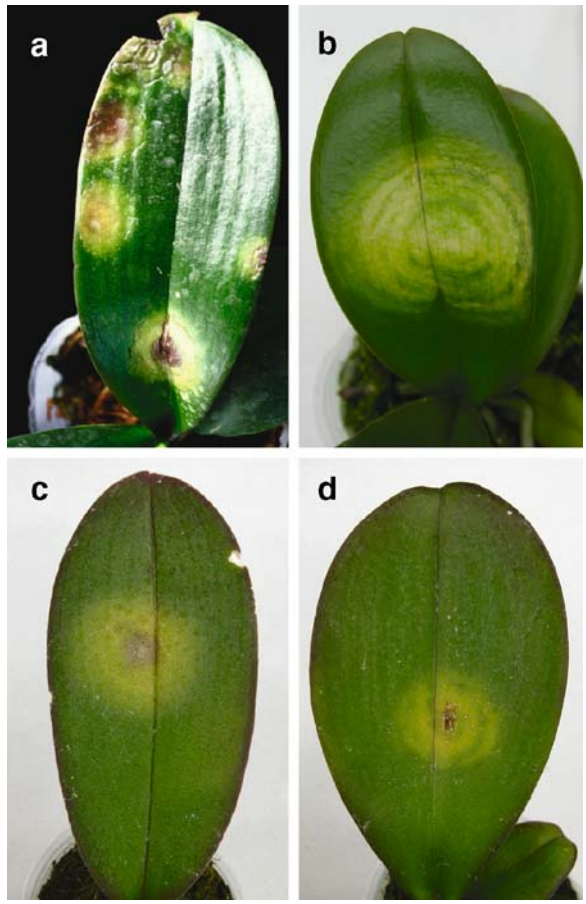

Fig. 1 *Phalaenopsis* orchid infected with Capsicum chlorosis virus-*Phalaenopsis* (CaCV-Ph). *Phalaenopsis* leaf showing large chlorotic spots with centric necrosis (**a**) and large chlorotic ringspot (**b**) and *Phalaenopsis* seedlings inoculated with CaCV-Ph showing a chlorotic spot with centric necrosis (**c**) and a chlorotic ringspot (**d**)

Electron microscopy

Leaves of diseased *Phalaenopsis* orchids from the fields, and *Datura stramonium* and *N. benthamiana* inoculated with 91-orchid-1 were used for ultrathin section preparation by the protocol described previously (Chen et al. 2005; Jan and Yeh 1995). Leaves were cut into small pieces, fixed in 2% glutaraldehyde in 0.1 M phosphate solution (pH 7.0) for 4 h, then in 2.5% osmium tetroxide at room temperature for an additional 2 h before dehydrating in a graded series of ethanol, following by embedding in LR White Resin

(Polysciences, Inc., Pennsylvania, USA). Ultrathin sections cut with a diamond knife and collected on formvar-coated, carbon-stabilized copper grids (75 mesh) were stained with 2% uranyl acetate for 15 min followed by 2% lead acetate for 1.5 min, and then examined with a JOEL 200 CX electron microscope (JOEL Ltd., Tokyo, Japan).

Host range and back-inoculation

To characterize the biological properties of 91-orchid-1, plants from twenty-three species representing three families were used for a host-reaction test. To confirm that 91-orchid-1 is indeed the causal agent for the disease, *Phalaenopsis* seedlings were inoculated with the virus. Inocula were prepared by propagating the virus in *C. quinoa* and grinding the infected leaves in buffer 4. All tested plants were inoculated once, except that *Phalaenopsis* seedlings were inoculated twice. The second inoculation was made three days after the first inoculation. Inoculated plants were grown in a greenhouse and observed daily for at least 45 days. For confirmation of the virus infection, both symptomatic and asymptomatic inoculated-plants were analyzed with indirect ELISA using the antiserum against 91-orchid-1 prepared in this study. In case of ambiguous results, extracts from the test plants were checked by inoculation to the systemic host *N. benthamiana*.

Purification of nucleocapsid protein and production of polyclonal antibody to 91-orchid-1 (CaCV-Ph)

Nucleocapsid protein (NP) of 91-orchid-1 was purified by the procedures described by Yeh et al. (1996) with minor modifications. Leaves of 91-orchid-1-infected *C. quinoa* were harvested 3–4 days after mechanical inoculation, ground into fine powder with liquid nitrogen, and blended with 3 vol (ml g^{-1}) TB buffer (0.01 M Tris–HCl, pH 8.0, containing 0.01 M sodium sulfite and 0.1% cysteine). The extracts were centrifuged at 15,300×*g* for 10 min. The supernatant was filtered through Miracloth (EMD Biosciences, Inc., California, USA) and stirred with 1% Triton X-100 at 4°C for 30 min. The solution was then centrifuged at 79,700×*g* for 2.5 h through a 20% sucrose cushion with TB buffer. The pellets were resuspended in 8 ml TBG buffer (TB buffer with 0.01 M glycine) per 100 g tissue and further purified by 35% (*w*/*w*) cesium sulfate isopycnic centrifugation at 83,500×*g* for 15 h. The opalescent zones containing viral nucleocapsids were collected, diluted five-fold with TBG buffer and further centrifuged at 163,600×*g* for 1 h. The purified nucleocapsids in the pellet were resuspended in 3 ml TBG buffer. The nucleocapsid proteins (NPs) were dissociated from the purified nucleocapsids and subjected to preparative 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and polyclonal antibody (PAb) against the NP of 91-orchid-1 was generated in a New Zealand white rabbit as described previously (Jan and Yeh 1995).

Immunoblotting

Immunoblotting was conducted according to the method described by Jan and Yeh (1995). Leaf tissues of mock-inoculated and virus-infected *C. quinoa* were ground in 3 vol (*v*/*w*) of dissociation buffer (100 mM Tris–HCl, pH 7.2, 2% β-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue, and 10 mM EDTA). The crude antigens in extracts were boiled and electrophoretically separated on a 12% SDS polyacrylamide gel and then transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad, California, USA). Subsequently, the membrane was preincubated with TSW buffer [10 mM Tris–HCl (pH 7.4), 0.9% NaCl, 0.25% gelatin, 0.1% Triton X-100, and 0.2% SDS] for 1 h, and then incubated with primary antiserum against 91-orchid-1 or WSMoV NP at 1:4,000 dilution in TSW buffer for 1 h, followed by alkaline phosphatase (AP) conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) for 1 h (1:5,000 dilution in TSW buffer) and colour developed by chromogenic substrates (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate paratoluidine salt in 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5). Extracts from leaves of uninfected plants and normal serum were used as controls.

ELISA

The procedure of indirect enzyme-linked immunosorbent assay (indirect ELISA) followed the method described by Clark and Adams (1977) with some modifications. Rabbit antisera for INSV, TSWV, WSMoV, PCFV, CMV, TuMV, *Zantedeschia mild mosaic virus* (ZaMMV), *Zantedeschia mosaic virus* (ZaMV), *Dasheen mosaic virus* (DsMV), *Carnation*

mottle virus (CarMV), *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV) and 91-orchid-1 were individually prepared. Clear Polystyrene microtitration plates (Greiner Bio-One, Frickenhausen, Germany) were coated with crude extracts diluted to 50-fold in coating buffer (0.05 M sodium carbonate, pH 9.6, 0.02% sodium azide). The rabbit antisera to each virus were used at 1:4,000 dilution in conjugate buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 and 0.2% ovalbumin) and followed by addition of alkaline phosphatase (AP) conjugated goat anti-rabbit IgG at 1:5000 dilution in conjugate buffer. The alkaline phosphatase substrate tablets (Sigma-Aldrich, St. Louis, MO, USA) were prepared in the substrate buffer (9.7% diethanolamine, and 0.02% sodium azide, pH 9.8) to a final concentration of 1 mg ml^{-1}. The level of reaction was recorded at 405 nm using a Labsystems Multiskan EX microplate reader (Labsystems, Vantaa, Finland) at 30 min after the addition of the substrate solution. Commercial ELISA kits for ORSV, CymRSV and CymMV (Agdia, Inc., Indiana, USA) were also used to react with crude extract from diseased *Phalaenopsis* orchids according to the manufacturer's protocol.

Molecular cloning and sequence analysis

Total RNA was extracted from leaves of *Phalaenopsis* orchid with chlorotic and necrotic spot symptoms, and 91-orchid-1-infected *C. quinoa* using the method described by Napoli et al. (1990). The degenerate primer pair gL3637 (5′-CCTTTAACAGT(A/T/G) GAAACAT-3′) and gL4435c (5′-CAT(A/T/G)GC(A/G)CAAGA(A/G)TG(A/G)TA(A/G)ACAGA-3′) (Fig. 4; Chu et al. 2001) for the amplification of the conserved region of the L genes of tospoviruses was used for reverse transcription-polymerase chain reaction (RT-PCR) to determine whether the virus 91-orchid-1 is a tospovirus. The strategies and primers used for RT-PCR, cloning and sequencing of the N gene and the 3′-end of the S RNA of 91-orchid-1 are presented in Fig. 5. The first-strand cDNA was synthesized with *Moloney murine leukemia virus* reverse transcriptase (M-MLV RT; Epicentre, Wisconsin, USA) at 42°C for 30 min and 37°C for another 30 min using downstream primers. PCR amplification was performed using *Taq* DNA polymerase with the first cycle at 93°C for 5 min, followed by 35 cycles at 93°C 1 min, 50°C for 1 min, and 72°C for 2 min, and final cycle at 72°C for 5 min (Jan et al. 2000). The RT-PCR products were cloned using pCRII-TOPO cloning kit (Invitrogen, California, USA) according to the instructions of the manufacturer. The recombinant clones were sequenced by automatic DNA sequencing method by ABI373 automated sequencer (Applied Biosystems, California, USA) at the Biotechnology Centre, National Chung-Hsing University, Taichung, Taiwan. The Gap programme of the SeqWeb (version 3.1.2, Accelrys, California, USA) was used to determine the degrees of the nucleic acid and amino acid identities between tospoviruses. The PROTDIST programme of PHYLIP version 3.63 (Department of Genetics, University of Washington, Washington, USA) was used to calculate the distance matrices of protein sequences. The nucleotide sequences of the L RNA of tospoviruses in GenBank used for alignment were WSMoV (AF133128), Peanut bud necrosis virus (PBNV, AF025538), TSWV (NC_002052), INSV (NC_003625), Melon yellow spot virus (MYSV, AB061774) and Tomato yellow fruit ring virus (TYFRV, AJ493271). The N gene sequences of tospoviruses used for comparison were WSMoV (NC_0038430), PBNV (U27809), TSWV (D13926), INSV (X66972), MYSV (AB024332), TYFRV (AJ493270), CaCV isolate 958 (AY036057), CaCV isolate AIT (NC_008301), CaCV isolate CP (DQ355974), Calla lily chlorotic spot virus (CCSV, AY867502), Watermelon bud necrosis virus (WBNV, AF045067), GRSV (L12048), *Tomato chlorotic spot virus* (TCSV, S54325), Chrysanthemum stem necrosis virus (CSNV, AF067068), Iris yellow spot virus (IYSV, AF001387), Peanut yellow spot virus (PYSV, AF013994), *Zucchini lethal chlorosis virus* (ZLCV, AF067069) and PCFV (AF080526).

Results

Symptoms and virus isolation

In November 2002, one *Phalaenopsis* plant bearing chlorotic ringspots measuring about 2.0 cm in diameter and some with 1.0 cm necrotic spots in the central region (Fig. 1a) and another plant bearing chlorotic ringspots (Fig. 1b) about 3.5 cm in diameter were tested by ELISA with antisera against INSV, TSWV, WSMoV, PCFV, CMV, TuMV, ZaMMV, ZaMV, DsMV, CarMV, TMV, ToMV, ORSV,

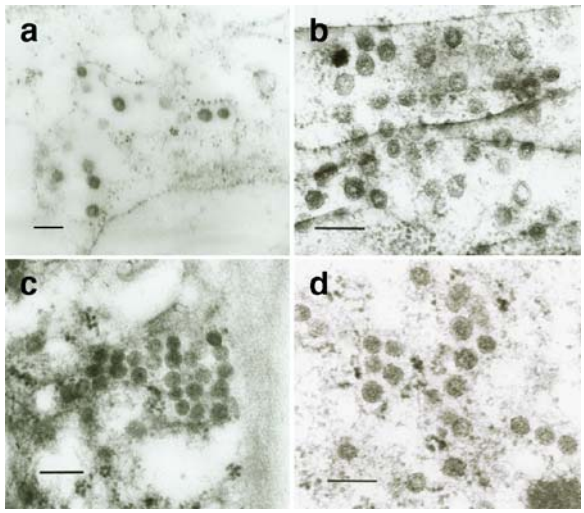

Fig. 2 Electron micrographs of viral particles from CaCV-Ph infected tissues. Virions in ultrathin sections from the *Phalaenopsis* orchid leaf showing large chlorotic spot with centric necrosis (**a**), and the CaCV-Ph-infected *Nicotiana benthamiana* (**b**), *Datura stramonium* (**c**) and *Phalaenopsis* seedling (**d**). *Bars* represent 200 nm

CymMV or CymRSV. These diseased leaves reacted positively only with WSMoV antiserum. A virus culture, 91-orchid-1, was then established in *N. benthamiana* and *C. quinoa* from leaves of *Phalaenopsis* orchids bearing chlorotic ringspots (Fig. 1a) through single-lesion isolation three times.

Host range and back-inoculation

From the twenty-three plant species mechanically inoculated, fifteen species were susceptible to the virus 91-orchid-1. Chlorotic local lesions were found on inoculated leaves of *N. glutinosa*, *N. occidentalis*, *N. tabacum* cv. Hicks, *N. tabacum* var. Samsun, *C. quinoa* and *C. murale*. Systemic infection of 91-orchid-1 was observed 6–10 days post-inoculation in *N. benthamiana*, *N. edwardsonii*, *N. tabacum* var. Xanthi, *N. tabacum* cv. Vam-Hicks, *N. rustica*, *Lycopersicon esculentum*, *Capsicum annuum* (red pepper), *C. annuum* var. *grossum* (sweet pepper) and *D. stramonium*. Symptoms on systemic hosts were mostly chlorotic ringspots or chlorotic spots initially which later developed into necrosis in the central region of spots. Infections were also confirmed by positive reactions of ELISA tests with antisera to 91-orchid-1. *Cucurbita pepo* var. *zucchini*, *Cucumis melo* (muskmelon), *C. melo var. conomon* Makino (oriental pickling melon), *Citrullus vulgaris* (watermelon), *Luffa acutangula* (loofah), *C. melo* var. *makuwa* Makino (melon) and *Lagenaria siceraria* (bottle gourd) were not infected with 91-orchid-1 as determined by symptom expression, ELISA and infectivity assay on *C. quinoa*.

To determine whether 91-orchid-1 was the causal agent for the disease observed in the field, the virus was back-inoculated onto five cultivars of *Phalaenopsis* orchid seedlings by mechanical inoculation. All the cultivars were found to be susceptible to the virus. At 10~14 days post-inocluation, *Phalaenopsis* seedlings inoculated with 91-orchid-1 showed symptoms of chlorotic ringspots similar to those occurring in nature (Fig. 1c,d). The chlorotic spots gradually covered the whole leaf and finally resulted in defoliation. The virus induced local infection on inoculated leaves of *Phalaenopsis* orchid plants. No systemic symptoms were observed on new leaves of the inoculated *Phalaenopsis* orchid plants during the 60 days of observation period. Infections were also confirmed by positive reactions of ELISA tests using antiserum of 91-orchid-1.

Electron microscopy

Electron microscopic examination of ultra-thin section of naturally infected *Phalaenopsis* orchids (Fig. 1a) revealed the presence of roughly spherical enveloped virion particles measuring 70–100 nm in diameter when stained with 2% uranyl acetate and 2% lead acetate (Fig. 2a). Similar-size particles were also observed in ultra-thin sections prepared from leaves of inoculated *Phalaenopsis* orchid (Fig. 2d). The morphology of observed particles was typically that of tospovirus. Tospovirus-like particles were also present in ultrathin sections of inoculated *N. benthamiana* (Fig. 2b) and *D. stramonium* (Fig. 2c).

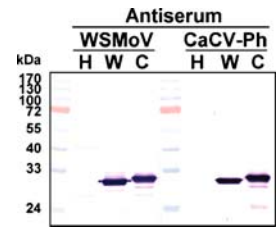

Fig. 3 Serological relationships of CaCV-Ph and *Watermelon silver mottle virus* (WSMoV) by western blotting. Antisera specific to nucleocapsid proteins (NPs) of WSMoV and CaCV-Ph were used to react with crude saps extracted from healthy *Chenopodium quinoa* (H) and leaves infected with WSMoV (W) or CaCV-Ph (C)

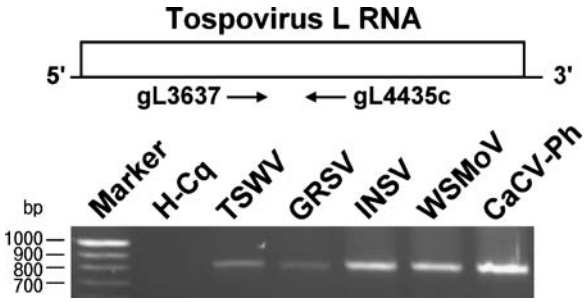

Fig. 4 Reverse transcription polymerase chain reaction (RT-PCR) of *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), WSMoV and CaCV-Ph using degenerate primer pairs designed from the conserved regions of the L RNA of tospoviruses

Serological analysis

The 91-orchid-1 orchid virus reacted with WSMoV antiserum in indirect ELISA test and revealed the presence of a tospovirus-like particle when examined by the electron microscope. To distinguish 91-orchid-1 from WSMoV and to detect the orchid virus, PAb against the NP of 91-orchid-1 was generated in rabbit. *Chenopodium quinoa* plants inoculated with WSMoV and 91-orchid-1 were tested by western blot. Both PAbs of 91-orchid-1 and WSMoV reacted strongly with the crude sap of 91-orchid-1 and WSMoV (Fig. 3). A protein band measuring about 30 kDa in the crude sap of 91-orchid-1-infected *C. quinoa* was observed in western blotting using antiserum against WSMoV or 91-orchid-1. Similarly, the 31 kDa NP of WSMoV reacted with antiserum either against WSMoV or 91-orchid-1.

Molecular cloning and sequence analysis

To determine the taxonomic relationships and characterize the genomic sequence of 91-orchid-1, one cDNA fragment around 800 base pairs (bp) was amplified from the total RNAs extracted from *Phalaenopsis* orchids showing chlorotic ringspots by RT-PCR using the degenerate primers, gL3637 and gL4435c, designed for amplifying the conserved region of L RNA of tospoviruses (Fig. 4)(Chu et al. 2001). The 0.8 kbp RT-PCR product was cloned, sequenced and analyzed using the Gap programme of the SeqWeb. The conserved region of L RNA of 91-orchid-1 shares 82.5, 83.8, 77.4, 75.9, 64.4 and 64.9% nucleotide identities and 97.7, 96.5, 91.1, 87.5, 67.3 and 67.6% amino acid identities with those of WSMoV, PBNV, MYSV, TYFRV, TSWV and INSV, respectively (Table 1). During this manuscript preparation, the complete genomic sequence of CaCV-AIT was published (Knierim et al. 2006). The conserved region of L RNA of 91-orchid-1 shares 86.9% nucleotide identity and 98.8% amino acid identity with those of CaCV-AIT (Table 1).

The sequence comparison of partial L RNAs indicated that 91-orchid-1 is a member of the genus *Tospovirus* closely related to WSMoV and PBNV. In order to more accurately determine the taxonomic status of 91-orchid-1, the nucleotide and amino acid sequences of N gene are required. The specific N gene primers of TSWV, GRSV, INSV, PCFV and WSMoV were used to amplify the N gene of 91-

Table 1 Comparisons of nucleotide and amino acid sequences of the conserved region of L protein of tospovirus isolate 91-orchid-1 (CaCV-Ph) with those of other tospoviruses available in database of GenBank

Virus[a]	91-orchid-1	CaCV-AIT	WSMoV	PBNV	MYSV	TYFRV	TSWV	INSV
91-orchid-1	–	86.9[b]	82.5	83.8	77.4	75.9	64.4	64.9
CaCV-AIT	98.8[c]	–	83.8	83.4	77.5	75.9	63.6	63.3
WSMoV	97.7	98.4	–	81.9	78.8	75.6	63.8	64.0
PBNV	96.5	97.7	96.9	–	76.6	77.9	65.1	64.9
MYSV	91.1	92.2	93.0	91.8	–	74.1	63.9	63.9
TYFRV	87.5	88.3	87.9	87.2	87.9	–	66.5	65.7
TSWV	67.3	68.1	67.7	67.7	67.7	67.7	–	74.9
INSV	67.6	68.0	66.5	67.6	66.1	66.5	83.3	–

[a] The GenBank accession numbers of tospovirus: *Capsicum chlorosis* virus-AIT (CaCV-AIT, NC_008302), *Watermelon silver mottle virus* (WSMoV, AF133128), Peanut bud necrosis virus (PBNV, AF025538), *Melon yellow spot virus* (MYSV, AB061774), Tomato yellow fruit ring virus (TYFRV, AJ493271), *Tomato spotted wilt virus* (TSWV, NC_002052) and *Impatiens necrotic spot virus* (INSV, NC_003625).

[b] Nucleotide identity (%)

[c] Amino acid identity (%)

orchid-1. Surprisingly, no DNA products were amplified by RT-PCR using the specific N gene primers of those tospoviruses from the total RNA isolated from 91-orchid-1-infected plants (Fig. 5a). The partial N gene was then amplified from 91-orchid-1 using primer pair N 3101/N3534c designed from the conserved sequence of the S RNA of the WSMoV serogroup tospoviruses (Fig. 5b; Chung 2002). An expected 0.45 kbp DNA band obtained from RT-PCR was cloned and sequenced and found to share 96.2% nucleotide identity with that of Capsicum chlorosis virus (CaCV) (accession number AY036057; McMichael et al. 2002). Another primer pair (FJJ2003–8: 5′-CATATATTCTAATATAAGTA-3′ and FJJ2003–10: 5′-GCACGCGGATCCAAGT CATCATATCAGAC-3′) was designed based on the partial N gene of 91-orchid-1 and the N gene of CaCV to amplify the full-length N gene of 91-orchid-1 (Fig. 5b). The resulting 0.9 kbp RT-PCR product was cloned and sequenced. The N ORF of 91-orchid-1 is 825 bp in length and encodes a predicted protein of 275 amino acids with a

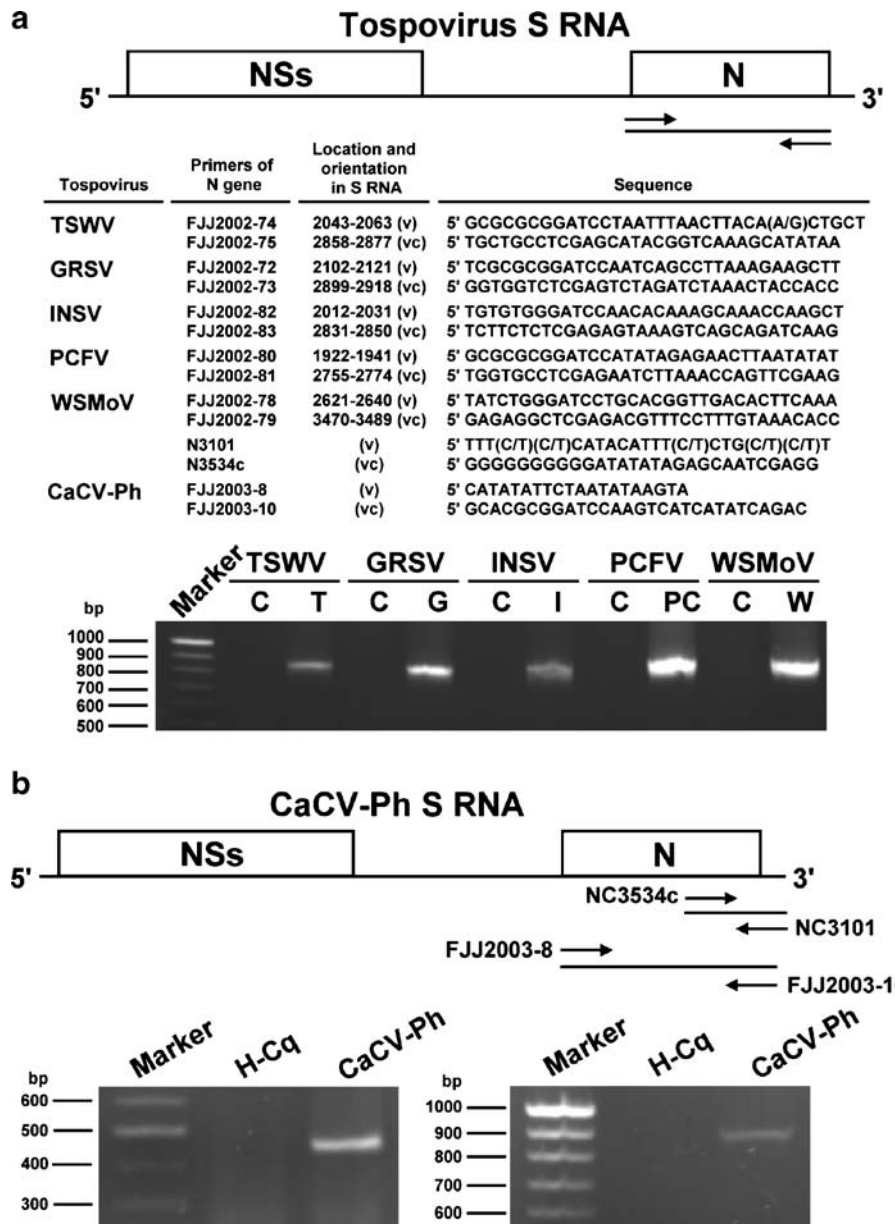

Fig. 5 RT-PCR of tospovirus genes. **a** RT-PCR of CaCV-Ph (C), TSWV (T), GRSV (G), INSV (I), Peanut chlorotic fan-spot virus (PCFV, PC) and WSMoV (W) using the specific primer pair designed for the N gene of TSWV, WSMoV, INSV, GRSV or PCFV. **b** RT-PCR of CaCV-Ph using the N gene degenerate primers of the viruses in WSMoV serogroup and the N gene specific primers for CaCV. Healthy *C. quinoa* (H-Cq) plants were used as controls

Table 2 Comparison of the nucleotide (nt) and deduced amino acid (aa) sequences of the nucleocapsid (N) gene of CaCV-Ph with those of other tospoviruses available in database of Genbank

Virus[a]	Sequence identity (%)	
	nt	aa
CaCV-958	96.1	97.5
CaCV-AIT	85.0	92.7
CaCV-CP	85.6	92.0
Gloxinia tospovirus HT	93.0	97.5
Gloxinia ringspot	85.9	93.1
WSMoV	77.5	84.4
PBNV	78.9	84.0
WBNV	77.5	81.1
CCSV	65.7	63.3
MYSV	62.6	59.6
IYSV	56.3	46.8
TYFRV	55.7	45.6
TSWV	46.0	34.3
GRSV	48.9	33.9
ZLCV	46.1	33.1
INSV	43.8	32.9
CSNV	46.6	32.8
TCSV	43.7	32.3
PCFV	43.2	28.0
PYSV	43.6	27.2

[a] Virus names: CaCV, Capsicum chlorosis virus; WSMoV, *Watermelon silver mottle virus*; PBNV, Peanut bud necrosis virus; WBNV, Watermelon bud necrosis virus; CCSV, Calla lily chlorotic spot virus; MYSV, Melon yellow spot virus; IYSV, Iris yellow spot virus; TYFRV, Tomato yellow fruit ring virus; TSWV, *Tomato spotted wilt virus*; GRSV, *Groundnut ringspot virus*; ZLCV, *Zucchini lethal chlorosis virus*; INSV, *Impatiens necrotic spot virus*; CSNV, Chrysanthemum stem necrosis virus; TCSV, *Tomato chlorotic spot virus*; PCFV, Peanut chlorotic fan-spot virus; PYSV, Peanut yellow spot virus

calculated molecular mass of 30.6 kDa. The nucleotide and predicted amino acid sequences of the 91-orchid-1 N gene share identities of 43.2 to 96.1% and 27.2 to 97.5%, respectively, with those of other tospoviruses available in Genbank whereas it shares highest nucleotide and amino acid identities of 85.0~96.1% and 92.0~97.5%, respectively, with those of CaCV isolates (Table 2). The phylogenetic analysis revealed that the NP relationship of 91-orchid-1 is more closely related to those of tospoviruses belonging to WSMoV serogroup including CaCV, PBNV, WSMoV, CCSV, WBNV, Gloxinia ringspot and Gloxinia tospovirus HT (Fig. 6, Table 2).

Discussion

Our results have demonstrated that the virus 91-orchid-1 causing chlorotic spots with centric necrosis on Phalaenopsis orchids is a tospovirus. N gene sequence analyses showed that 91-orchid-1 and the other virus, Tospo-2, isolated from diseased Phalaenopsis orchids showing large chlorotic ringspot (Fig. 1b) share 99.3% amino acid identity (data not shown) and they share high nucleotide and amino acid sequences identities with those of CaCV.

Comparison of the N gene sequences of the WSMoV serogroup of tospoviruses showed that 91-orchid-1 shared 85.0~96.1% nucleotide identity and 92.0~97.5% amino acid identity with those of CaCV infecting tomato and capsicum in Australia (isolate 958; McMichael et al. 2002), Thailand (isolate AIT) (Premachandra et al. 2005), peanut in southern China (isolate CP; Chen et al. 2007), Gloxinia tospovirus HT (accession No. AF059578; Hsu et al. 2000) and Gloxinia ringspot virus (accession No. AY312061) (Table 2). Based on the N gene sequence analyses, these four viruses identified in different countries infecting various crops should be classified in the same tospovirus species belonging to WSMoV serogroup (Fig. 6). Therefore, we have designated the name CaCV-Ph for this Phalaenopsis orchid-infecting virus.

Capsicum chlorosis virus (CaCV) was first reported to infect tomato and capsicum plants in Australia (McMichael et al. 2002) and Thailand (Premachandra et al. 2005). Recently, the virus caused great losses of peanut production in southern China (Chen et al. 2007). The host range of CaCV-Ph was different from that of CaCV-958 isolated from Australia. *Capsicum annuum* inoculated with CaCV-Ph showed necrotic ringspots and deformations on both inoculated and systemic leaves, and plants eventually wilted. However, isolate 958 caused mottling on systemic leaves of *C. annuum* and did not show any symptoms on inoculated leaves. *Lycopersicon esculentum* infected by CaCV-Ph or CaCV-958 showed necrotic spots systemically, but only CaCV-Ph caused chlorotic or necrotic spots on inoculated leaves. Although *N. glutinosa* was a local lesion host for CaCV-Ph and CaCV-958, *N. rustica* was a systemic host only for CaCV-Ph. The comparison of the host ranges helped to find out certain differences not only among CaCV isolates but also the natural hosts under greenhouse conditions.

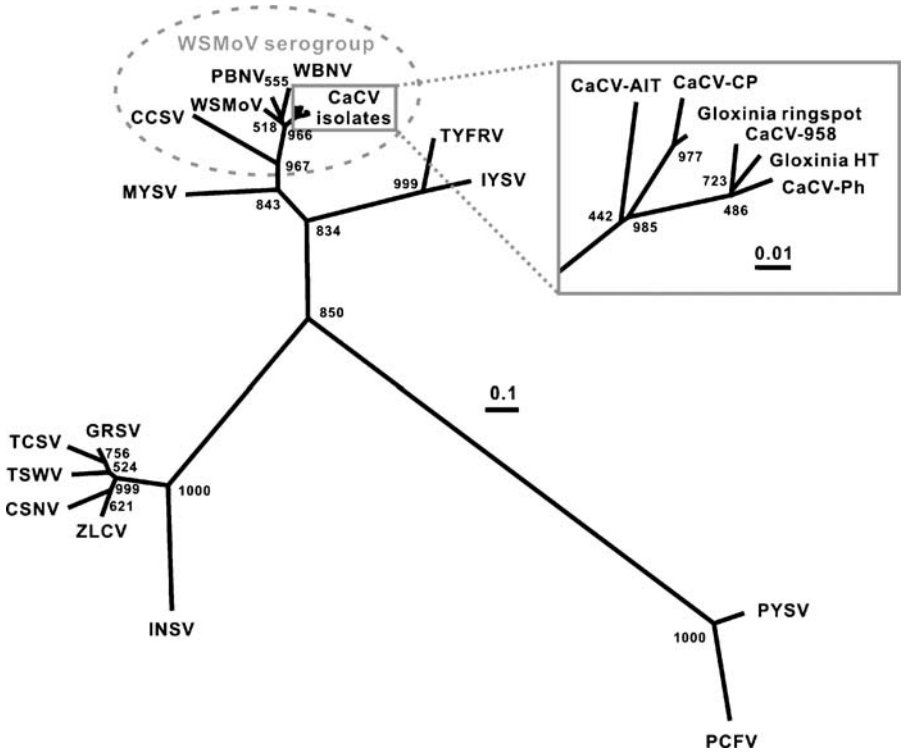

Fig. 6 Phylogenetic relationships of the NPs of CaCV-Ph and other tospoviruses. The scale for genetic distances is indicated at the middle right. Sequence sources are the same as in Table 2. Phylogenetic analysis was conducted using the Clustal-X and PHYLIP package version 3.63 (by J. Felsenstein and the University of Washington, Washington, USA). The dendrogram was produced using the neighbour-joining algorithm with 1,000 bootstrap replicates

CaCV-Ph causes symptoms of chlorotic ringspots on *Phalaenopsis* orchids distinct from those caused by CymMV, ORSV, and CMV. CaCV-Ph was first classified in *Tospovirus* group based on virus morphology, serological reaction and partial L gene sequence analyses. Without the N gene sequence, these characteristics cannot be used to distinguish CaCV-Ph from WSMoV. However, CaCV-Ph can infect tobacco, tomato, hot pepper and sweet pepper but not watermelon, melon and any other tested cucurbits. These results indicate that CaCV-Ph has host range different from WSMoV, a tospovirus infecting *Cucurbitaceae* crops in Taiwan.

Thrips palmi was reported to be the vector of the members of WSMoV serogroup (WSMoV, PBNV, and CCSV), MYSV and TSWV (Yeh et al. 1992; Vijayalakshmi 1994; Chen et al. 2005; Jan et al. 2003; Kato et al. 1999; Moyer 2000). Recently, CaCV was shown to be transmitted by *T. palmi* (Persley et al. 2006) and tomato thrips (*Ceratothripoides claratris*; Premachandra et al. 2005). We have demonstrated ability of *T. palmi* to transmit WSMoV (Yeh et al. 1992) and CCSV (Chen et al. 2005) isolated from Taiwan. However, our transmission experiments conducted under greenhouse conditions showed that *T. palmi* was not capable of transmitting CaCV-Ph (unpublished data). Moreover, tomato thrips (*C. claratris*) which has been shown to transmit CaCV in Thailand (Premachandra et al. 2005) has not been found in Taiwan yet. Therefore, vectors for transmission of CaCV-Ph in Taiwan remain to be investigated.

Members of WSMoV serogroup can damage the vegetable and fruit crops and are prevalent in tropics and subtropics, including WSMoV and CCSV in Taiwan (Yeh et al. 1992; Chen et al. 2005), PBNV and WBNV in India (Reddy et al. 1992; Singh and Krishnareddy 1996), and CaCV in Australia, Thailand and China (McMichael et al. 2002; Pongsapich and Chiemsombat 2002; Jones 2005; Chen et al. 2007). The new CaCV-Ph has caused serious damage on orchid production in Taiwan. The PAb generated for detection of CaCV-Ph in this study should be useful for detecting the virus in the nurseries and for obtaining virus-free plants for propagation.

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References

Chen, C. C., Chen, T. C., Lin, Y. H., Yeh, S. D., & Hsu, H. T. (2005). A chlorotic spot disease on calla lilies (*Zantedeschia* spp.) is caused by a tospovirus serologically but distantly related to *Watermelon silver mottle virus*. *Plant Disease*, *89*, 440–445.

Chen, C. C., & Chiu, R. J. (1996). A tospovirus infection peanut in Taiwan. *Acta Horticulturae*, *431*, 57–67.

Chen, T. C., Hsu, H. T., & Yeh, S. D. (1998). *A new tospovirus-like virus isolated from orchid*. (Abstracts of papers and poster presentations presented at the 4th International Symposium on Tospoviruses and Thrips in Floral and Vegetable Crops, Netherlands).

Chen, K., Xu, Z., Yan, L., & Wang, G. (2007). Characterization of a new strain of Capsicum chlorosis virus from peanut (*Arachis hypogaea* L.) in China. *Journal of Phytopathology*, *155*, 178–181.

Chu, F. H., Chao, C. H., Chung, M. H., Chen, C. C., & Yeh, S. D. (2001). Completion of the genome sequence of *Watermelon silver mottle virus* and utilization of degenerate primers for detecting tospoviruses in five serogroups. *Phytopathology*, *91*, 361–368.

Chung, M. H. (2002). Assessment of the variability of different isolates of *Watermelon silver mottle virus* occurring in Taiwan. Master Thesis, National Chung Hsing University.

Clark, M. F., & Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, *34*, 475–483.

Hsu, H. T., Ueng, P. P., Chu, F. H., Ye, Z. H., & Yeh, S. D. (2000). Serological and molecular characterization of a high temperature-recovered virus belonging to tospovirus serogroup IV. *Journal of General Plant Pathology*, *66*, 167–175.

Jan, F. J., Chen, T. C., & Yeh, S. D. (2003). Occurrence, importance, taxonomy and control of thrips-borne tospoviruses. In H. Huang, & S. N. Acharya (Eds.) *Advances in Plant Disease Management* (pp. 391–411). India: Research Signpost.

Jan, F.-J., Fagoaga, C., Pang, S. Z., & Gonsalves, D. (2000). A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *Journal of General Virology*, *81*, 2103–2109.

Jan, F.-J., & Yeh, S. D. (1995). Purification, in situ localization, and comparative serological properties of passionfruit woodiness virus-encoded amorphous inclusion protein and two other virus proteins. *Phytopathology*, *85*, 64–71.

Jones, D. R. (2005). Plant viruses transmitted by thrips. *European Journal of Plant Pathology*, *113*, 119–157.

Kato, K., Hanada, K., & Kameya-Iwaki, M. (1999). Transmission mode, host range and electron microscopy of a pathogen causing a new disease of melon (*Cucumis melo*) in Japan. *Annals of the Phytopathological Society of Japan*, *65*, 624–627.

Knierim, D., Blawid, R., & Maiss, E. (2006). The complete nucleotide sequence of a capsicum chlorosis virus isolate from *Lycopersicum esculentum* in Thailand. *Archives of Virology*, *151*, 1761–1782.

Ko, N. J. (1988). Cytological identification of *Cucumber mosaic virus* infecting *Phalaenopsis*. *Proceedings of the National Science Council, Republic of China*, *12*, 46–51.

Ko, N. J., Zettler, F. W., & Edwardson, J. R. (1988). Immunocytochemical identification of Cymbidium mosaic and Odontoglossum ringspot viruses in orchid tissues. *Acta Horticulturae*, *234*, 299–306.

Law, M. D., & Moyer, J. W. (1990). A tomato spotted wilt-like virus with a serologically distinct N protein. *Journal of General Virology*, *71*, 933–938.

Lawson, R. H., & Brannigan, M. (1986). Virus disease of orchids. *Handbook of Orchid Pests and Diseases* (pp. 2–49). Florida: American Orchid Society.

Lawson, R. H., & Hsu, H. T. (1995). Orchid. In G. Loebenstein, R. H. Lawson, & A. A. Brunt (Eds.) *Virus and virus-like diseases of bulb and flower crops* (pp. 409–420). UK: Wiley.

Lesemann, D. E. (1977). Long, filamentous virus-like particles associated with vein necrosis of *Dendrobium phalaenopsis*. *Phytopathologische Zeitschrift*, *89*, 330–339.

McMichael, L. A., Persley, D. M., & Thomas, J. E. (2002). A new tospovirus serogroup IV species infecting capsicum and tomato in Queensland, Australia. *Australasian Plant Pathology*, *31*, 231–239.

Moyer, J. W. (2000). Tospoviruses. In R. Hull (Ed.) *Encyclopedia of Microbiology* (pp. 592–597). London: Academic Press.

Napoli, C., Lemieux, C., & Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*, *2*, 279–289.

Pang, S. Z., Slightom, J. L., & Gonsalves, D. (1993). The biological properties of a distinct tospovirus and sequence analysis of its S RNA. *Phytopathology*, *83*, 728–733.

Persley, D. M., Thomas, J. E., & Sharman, M. (2006). Tospoviruses-an Australian perspective. *Australasian Plant Pathology*, *35*, 161–180.

Pongsapich, P., & Chiemsombat, P. (2002). *Characterization of tospovirus infecting tomatoes in Thailand revealed the presence of serogroup IV-tospovirus but not serogroup I-tomato spotted wilt virus*. Paper presented at the 1st International Conference on Tropical and Subtropical Plant Diseases, Chiang Mai, Thailand.

Premachandra, W. T. S. D., Borgemeister, C., Maiss, E., Knierim, D., & Poehling, H. M. (2005). *Ceratothripoides claratris*. , a new vector of a Capsicum chlorosis virus isolate infecting tomato in Thailand. *Phytopathology*, *95*, 659–663.

Reddy, D. V. R., Ratna, A. S., Sudarshana, M. R., Poul, F., & Kumar, I. K. (1992). Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis-hypogaea* L.) in India. *Annals of Applied Biology*, *120*, 279–286.

Singh, S. J., & Krishnareddy, M. (1996). Watermelon bud necrosis: A new tospovirus disease. *Acta Horticulturae*, *431*, 68–77.

Vijayalakshmi, K. (1994). Transmission and ecology of *Thrips palmi* Karny, the vector of peanut bud necrosis virus. Dissertation, Andhra Pradesh Agricultural University.

Yeh, S. D., Chao, C. H., Cheng, Y. H., & Chen, C. C. (1996). Serological comparison of four distinct tospoviruses by polyclonal antibodies to purified nucleocapsid proteins. *Acta Horticulturae*, *431*, 122–134.

Yeh, S. D., Lin, Y. C., Cheng, Y. H., Jih, C. L., Chen, M. J., & Chen, C. C. (1992). Identification of tomato spotted wilt-like virus infecting watermelon in Taiwan. *Plant Disease*, *76*, 835–840.

Zettler, F. W., Ko, N. J., Wisler, G. C., Elliott, M. S., & Wong, S. M. (1990). Viruses of orchids and their control. *Plant Disease*, *74*, 621–625.