Identification of Squash leaf curl Philippines virus on Benincasa hispida in Taiwan

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ABSTRACT


During a disease survey of wax gourd (Benincasa hispida Cogn.) in Taiwan, virus-like symptoms of yellowing, vein enation, rugose mosaic and leaf curling were observed and further characterized. A naturally infected wax gourd sample (Wg1) was collected from experimental field at Beidou, Changhua County, Central Taiwan. The plant was tested negative with indirect ELISA using the antisera against eight RNA viruses commonly found in cucurbits, including Cucurbit aphid-borne yellows virus, Cucumber green mottle mosaic virus, Cucumber mosaic virus, Melon vein-banding mosaic virus, Papaya ringspot virus-watermelon type, Watermelon silver mottle virus and Zucchini yellow mosaic virus. However, it showed positive to a geminivirus, tentatively named isolate of Squash leaf curl virus Wg1 (SqLCV-Wg1), with polymerase chain reaction (PCR) using primer pair that is specific to begomovirus genomes. After cloning and sequencing, the PCR product was identified to be 1116 nucleotides (nt) in length, encompassing 126, 771, and 219 nt of the AV2, CP, and AC3 gene coding regions, respectively. Sequence alignment with the corresponding regions of other begomoviruses revealed that SqLCV-Wg1 is most closely related to Squash leaf curl Philippines virus, with percent nucleotide identities of 97.6, 97.7, and 93.8 for AV2, CP, and AC3 genes, respectively. The CP gene of SqLV-Wg1 was further cloned into the vector pET21d(+), which was then used to express proteins in Escherichia coli BL21(DE3) and to produce specific antiserum for future diagnosis purposes. This is the first record of cucurbitaceous plants infected with Squash leaf curl virus in Taiwan.

Key words: cucurbit, Benincasa hispida, Squash leaf curl virus, identification

INTRODUCTION

Benincasa hispida Cogn., commonly known as wax gourd or white gourd, is a fast-growing, long-season, warm-climate vegetable. The plant produces edible fruits with green peel and white pulp containing numerous small seeds, which can grow up to 50 pounds. The unopened fruits can be stored in a cool place for months and used later in winter. Thus, this gourd is called Tong Qwa, meaning “Winter Melon” in Chinese. Wax gourd is not only a flavor vegetable at the low price, but could also reduce weight for people of obesity and prevent development of ulcers ⁵. According to Taiwan Agriculture Annual Report in 2005, the growing area of wax gourd has
been increased to about 1500 hectares, among which 600 hectares concentrating in Changhua County. In recent years, wax gourd cultivation has been increasing in the southern and central Taiwan.

During a disease survey of wax gourd in Taiwan, several diseased plants with virus-like symptoms such as yellowing, vein enation, rugose mosaic and leaf curling were discovered in fields at Beidou, Changhua County, Central Taiwan. The diseased plants showed growth decline. It has been documented in Taiwan that wax gourd is infected by several RNA viruses, including Cucurbit aphid-borne yellows virus (CABYV), Cucumber green mottle mosaic virus (CGMMV), Cucumber mosaic virus (CMV), Melon vein-banding mosaic virus (MVbMV), Papaya ringspot virus W type (PRSV-W), Tomato spotted wilt virus (TSWV), Watermelon silver mottle virus (WSMoV), and Zucchini yellow mosaic virus (ZYMV)\(^{(11,12)}\). However, none of these RNA viruses could induce the symptoms similar to those on the diseased wax gourd plants described as above.

The first case of DNA virus infecting cucurbits was discovered in squash leaf curl disease\(^{(1)}\) in Philippine in 1977. The causal agent, Squash leaf curl virus, was further identified through nucleotide sequence analysis in 1991\(^{(14)}\). Several begomoviruses have been reported in the Southeast Asia regions, such as “Squash leaf curl virus-Vietnam” and “Loofa yellow mosaic virus-Vietnam” infecting the cucurbits in Vietnam\(^{(19)}\), as well as “Tomato leaf curl virus” causing a yellow leaf disease of cantaloupe and wax gourds from Thailand\(^{(19)}\). As for DNA viruses on cucurbits\(^{(4,5,10)}\), the geminivirus infecting wax gourds has not yet been reported in Taiwan.

In this study, we described the identification, molecular characterization, and phylogenetic analysis of a SqLCV associated with the new disease in wax gourds in Taiwan. This pathogen was tentatively named Squash leaf curl virus Wg1 isolate (SqLCV-Wg1). The specific antiserum against SqLCV-Wg1 was also prepared for rapid diagnosis of SqLCV-Wg1. This is the first report associated with the detection and molecular characterization of a SqLCV in Taiwan.

**MATERIALS AND METHODS**

Field observation and virus source

In the summer of 2005, a disease survey of wax gourd was conducted in the experimental field at Beidou, Changhua County, Central Taiwan. The naturally infected plants showing virus-like symptoms were collected and tested by indirect ELISA using specific antisera against CABYV, CGMMV, CMV, MVbMV, PRSV-W, TSWV, WSMoV or ZYMV. Virus cultures were maintained in wax gourd plants. Inoculations were done with whitefly vector [Bemisia tabaci (Gennadius)].

**Polymerase chain reaction (PCR)**

The forward and reverse specific primers gen-793-f (5’ gttttcatggtctacgggt 3’) and gen-793-r (5’ aaccacaacctgcggaaag 3’)\(^{(3,12)}\) for amplification of geminivirus CP gene were used in PCR. Total DNAs were extracted from infected wax gourds using commercial kit (Viogene, Plant DNA Extraction Miniprep System, TW) according to the manufacturer’s instructions. Amplification of the DNA was performed in a volume of 50 μl of reaction mixture containing: 3 μl DNA template, 5 μl 10x PCR buffer, 4 μl of 2.5 mM dNTP, 0.1 μl Ex-Taq Polymerase (Takara Shuzo Co., Shiga, Japan), 2.5 μl of 20 mM gen-793-f and 2.5 μl of 20 mM gen-793-r primers. Thirty-five cycles of PCR were performed using thermal cycler (Biometra Uno-thermoblock, Biometra biomedizinische Analytik GmbH) with denaturation at 94 °C for 2 min, primer annealing at 58 °C for 1 min, and DNA synthesis at 72 °C for 1-min 20-sec followed by a final elongation step at 72 °C for 8 min\(^{(19)}\). Eight μl of amplified products were analyzed by electrophoresis in 1 % agarose gel in TAE buffer. Bands were visualized by ethidium bromide staining.

**Cloning and sequence analyses**

The amplified DNA products were cloned into dT&A cloning vector and then transformed to E. coli. ECOS competent cell (Yeastern Biotech Co., Ltd,TW) according to the instructions provided by the manufacturer. DNA sequences of the correct clones were confirmed by an automatic DNA sequencing system ( ABI 3730 XL DNA Analyzer, Applied Biosystems, CA). The deduced amino acid sequences were analyzed using the Translate program of the GCG software (GCG Wisconsin Package, Accelrys Inc., San Diego, CA). MegAlign program (DNASTAR, Inc) was used for pairwise and multiple sequence alignments of DNA or protein, and to create phylogenetic trees as well as reports and tables showing the numerical data underlying the comparisons. Sequences of other related cucurbit-infecting begomoviruses for comparisons were obtained from the EMBL Database under the following accession numbers: Squash leaf curl Philippines virus, AB085793.1; Squash leaf curl China virus, AF509741.1; Chinese squash leaf curl virus, S77090.1; Tomato leaf curl New Delhi virus, U15015.2, as described by Kon et al\(^{(20)}\).

**Expression of coat protein (CP) gene**
The specific primers, gen-exp-f (5'-acctgccaagcagcccaggatatt 3') and gen-exp-r (5' cccgatatcagtaataatgat 3'), corresponding to the 5'- and 3'-termini of CP gene of SqLCV-Wg1 isolate was used to amplify the CP gene. Primers also contained NcoI and BamHI restriction sites as non-template overhangs for cloning purposes. The reaction was carried out in 35 cycles of 45sec denaturation at 94°C, 45 sec annealing at 55°C and 1 min 20 sec elongation at 72°C. The PCR products were directly cloned to dT&A cloning vector. The plasmid pET21d (+) was used for subcloning the PCR product above-mentioned, and expression of the CP gene of SqLCV-Wg1 (Wg1-CP) in E. coli BL21 (DE3) in Luria-Bertani (LB) medium (21). A 5-ml overnight culture of BL21(DE3) cells containing plasmids was added to 50 ml of LB and the culture was grown to an optical density at 600 nm (OD600) of 0.6, and then induced with isopropyl-1-thio-beta-D-galactoside (IPTG, Serva, Heidelberg, Germany) in a final concentration of 0.2 mM. The culture was then incubated for 3 h at 37°C. Bacterial cells were harvested by centrifugation and stored at -80°C until used.

Preparation of CP-enriched fractions

Wg1-CP gene was partially purified from 125 ml of the bacterial culture according to the procedure described. The pellet and supernatant fractions obtained from centrifugations of bacterial cells were sonicated and partially resuspended in a small volume of phosphate-buffered saline (PBS) or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoretic buffer, and subjected to further testing. The major portion of fusion proteins was in the pellet fractions. The pellets were re-suspended and further purified using His Excellulose Spin Kit (Yeastern Biotech Co., Ltd) as instructed by the manufacturer.

Antiserum production

The antiserum against the bacterially expressed Wg1-CP was prepared in a New Zealand White rabbit given three subcutaneous injections of 1 mg recombinant protein at three weekly intervals. The purified proteins were emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for the following two subsequent injections. The recombinant protein was soluble in both types of adjuvant. The rabbit were bled 5 weeks after the last injection (16). The serum fractions were collected and stored at -20°C until required.

Indirect enzyme-linked immunosorbent assay (I-ELISA)

Clear Polystyrene 96-well plates were coated with crude extracts diluted 1:10 and 100 fold in coating buffer (0.05 M sodium carbonate, pH 9.6, containing 0.01 % sodium azide). The above antiserum specific to Wg1-CP at 1:250, 1:500, 1:1000 and 1:2000 dilution in conjugate buffer (phosphate-buffered saline containing 0.05 % Tween 20, 2% polyvinylpyrrolidone-40, and 0.2% ovalbumin) were used. Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase (AP) conjugate (Kirkegaard & Ferry Laboratories, Gaithersburg, MD) was diluted to 1:5000 in conjugate buffer. Substrate solution was prepared by dissolving ρ-nitrophenyl phosphate tablets (Sigma Chemical, St. Louis, MO) in substrate buffer (97 ml of diethanolamine and 0.2 g of sodium azide per liter, pH 9.8) to a final concentration of 1 mg/ml (8). Absorbance at 405 nm (A405nm) was recorded using a Thermo Labsystems Multiskan reader (Thermo electron corr. U.S.A.) 30 min after the addition of substrate. The naturally infected wax gourd plants showing systemic mild mottling and leaf curl symptoms were collected as samples.

RESULTS

Field observation and virus isolation

From July to September 2005, eleven wax gourd plants including 2 resistant varieties (TVI4204) which are resistant to CABYV, CGMMV, CMV, MVbMV, PRSV-W, TSWV, WSMoV and ZYMV and 9 susceptible varieties (TV7296, TVI10688, TVI10689 and TVI11577) with a novel type of virus-like symptoms including mottling, yellowing, vein enation, rugose mosaic, or leaf curling were observed and collected. Out of them a wax gourd plant (Wg1) with systemic mild mottling and leaf curl (Fig. 1) but negatively reacted with antiserum against CABYV, CGMMV, CMV, MVbMV, PRSV-W, TSWV, WSMoV and ZYMV was collected for further analyses.

Polymerase chain reaction (PCR)

After examination of several RNA viruses frequently found in cucurbits, it was inferred that DNA viruses, such as geminiviruses, might be the causal agents in Wg1 plant. Therefore, total DNAs extracted from Wg1 was assayed by PCR with the specific primers devised from CP gene of bipartite geminiviruses. Specific fragments (1.1 kb) (Fig. 2) were amplified, which demonstrated that Wg1 plant was infected by geminiviruses. To further characterize the virus associated with the diseased wax gourd plants, the specific
PCR products were cloned. Three clones were selected to sequence and analyze. It revealed that the PCR product consisted of 1116 nucleotides spanning 5',3' - terminal 126 nucleotides of the AV2 gene, the entire 771 nucleotides of the CP coding region (257 aa), and the 3' - terminal 219 nucleotides of AC3 gene (73 aa).

**Sequence analysis**

BLAST analysis revealed that the new virus is closely related to *Squash leaf curl virus* (Fig. 3), and it was tentatively named SqLCV-Wg1. To further analyze phylogenetic relationships between SqLCV-Wg1 and other

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**Fig. 1. Symptoms of wax gourd (Wg1) infected by *Squash leaf curl virus*.** The leaves and shoot tip of a wax gourd plant (Wg1) were shown with systemic mild mottling, vein clearing, and leaf curling (as arrowed).

**Fig. 2. Electrophoreses of polymerase chain reaction (PCR) products corresponding to the coat protein (CP) gene of SqLCV-Wg1 amplified using specific primer (gen-793-f, gen-793-r).** Total DNAs extracted from infected wax gourds were used as templates for PCR with CP gene-specific primer pair as described previously. The amplified DNA products were analyzed by electrophoresis through a 1% agarose gel, and observed on a UV-light box by staining with ethidium bromide. Lanes 1-3, products of PCR amplification of three independent samples. The expected size of SqLCV-Wg1 CP gene, about 1.1 kb in length, is indicated by the arrow on the right. Lane M, DNA standard ladder.

**Fig. 3. The difference of amino acid sequences between SqLCV-Wg1 partial gene and *Squash leaf curl Philippines virus* (Accession No. AB085793).** The region corresponding to the CP gene is indicated on the alignment. Amino acids are presented with the single-letter code. Identical amino acids were shown with white letters on black background. The consensus amino acid sequence between the two sequences is shown under the alignment.
begomoviruses, the program MegAlign was used for pairwise and multiple sequence comparisons. The percent sequence identities for nucleotide sequences of entire CP gene between SqLCV Wg1 and other begomoviruses ranged from 94.6 % (with Tomato leaf curl virus, AY691899; AY691902, Squash leaf curl China virus, AY184487) to 97.7 % (with Squash leaf curl Philippines virus, AB085793), and those for the amino acid ranged from 94.9 % (with Tomato leaf curl virus, AY691899) to 98.0 % (with Squash leaf curl Philippines virus, AB085793) (Table 1). The results suggested that SqLCV-Wg1 should be classified as an isolate of Squash leaf curl Philippines virus.

Expression and purification of Wg1-CP

To produce antiserum against the CP of SqLCV-Wg1 for diagnosis purposes, a clone containing the CP gene of SqLCV-Wg1 was used for further experiments. After digestion with restriction enzymes NcoI and BamHI, the resulting DNA fragment carrying the CP gene was ligated into pET21d (+) plasmid (Fig. 4) and transformed E. coli BL21 (DE3). After optimization of expression conditions, including time, temperature, and the concentration of IPTG inducer, the cells were harvested and the expected protein (31 kDa) was demonstrated by SDS-PAGE and Western blotting analysis (Fig. 5A, 5B). SDS-PAGE showed a strong band at a position corresponding to the expected molecular weight of about 31 kDa, consisting of the complete Wg1-CP with 6xHis tag. However, the major portion of fusion proteins was in the pellets. Therefore, the pellets were further suspended and the target proteins were purified using His Excellulose Spin Kit (Yeaster Biotech Co., Ltd). Polyclonal antiserum to the recombinant Wg1-CP was obtained from bleeds taken 3 weeks after the last injection. The anti-Wg1-CP sera had titres of 1:500 when tested in an I-ELISA with purified fused proteins in microtitre plates (data not shown).

Indirect enzyme-linked immunosorbert assay (I-ELISA)

Positive results were observed in indirect ELISA of crude extracts prepared from naturally infected wax gourd plants from fields at Beidou, Changhua County by using the antiserum against recombinant SqLCV-Wg1 CP. The healthy wax gourd plants tested negative in all the assays. The $A_{405nm}$ reading of specific reaction was about five times

<table>
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<th>Accession No.</th>
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Nt : nucleotide    aa : amino acid.
that of negative control (Fig. 6). The antiserum diluted 250 and 500 fold was proven useful in indirect-ELISA test.

**DISCUSSION**

This study demonstrated that a viral disease on wax gourds occurred in Taiwan, and its has not yet recorded previously. Eight RNA viruses frequently observed in cucurbits were not detected in these symptomatic wax gourd plants. Through cloning and nucleotide sequence analyses, the provisional identity of the causal pathogen was determined to be an isolate of *Squash leaf curl Philippines virus* (1), designated as SqLCV-Wg1.
It has been reported that the pathogen causing yellow leaf disease on cantaloupe and wax gourds in Thailand was ToLCV or closely related strains. Although there were different symptoms between induced by ToLCV and SqLCV, it is difficult to distinguish Squash leaf curl Philippines virus from other Begomovirus members by serological assays. The result of indirect ELISA indicated that the concentration of SqLCV viros in infected tissue is not high, so the optimal antigen dilution is suggested to be 1-10 fold. On the other hand, the optimal antiserum titer is 250-500 fold. In this condition, we could reliably detect the existence of Squash leaf curl virus in cucurbitaceous plants.

The nucleotide sequence of CP gene of SqLCV-Wg1 is closely related to Squash leaf curl Philippines virus (97.7.0%); Squash leaf curl China virus [K] segment DNA-A (97.3%); Squash leaf curl China virus [B] segment DNA-A (96.8%); Squash leaf curl China virus DNA-A (96.1%); Chinese squash leaf curl virus (96.1%); Squash leaf curl China virus [Pumpkin :Coimbatore] (94.6%); and Tomato leaf curl virus (94.6%) in partial nucleotide sequence comparisons (Table 1). A similar analysis was applied to the deduced amino acid sequences of the putative ORF products (Table 1). Through sequence analysis, it was found that SqLCV-Wg1 is most closely related to Squash leaf curl Philippines virus (accession number AB085793). The significant sequence identity of the partial genomic DNA of SqLCV-Wg1 is compared to other distinct geminivirus species in the genus Begomovirus.

This is the first record of cucurbits infected with Squash leaf curl virus in Taiwan. For future studies, it is necessary to investigate the natural host range of SqLCV-Wg1, the epidemiology, the development and spread of the disease by whitefly vectors, and methods to decrease the population density of the vectors and the incidence of the disease in the field. The sequence information and serological tools provided in this study would be helpful in the understanding and the management of the new disease.

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structure and alteration of expression interaction alpha 1 in *Escherichia coli*. Gene 58: 77-86.

摘 要

廖吉彥’’、胡仲祺’’、林子凱’’、張清安’’、鄭汀欽’’。2007. 感染冬瓜的南瓜壓巂病之鑑定. 植病會刊 16: 11-18. (’’台中縣霧峰鄉行政院農業委員會農業試驗所植物病理組；’’國立中興大學生物科技所；’’台中縣霧峰鄉行政院農業委員會農業試驗所園藝組；’’聯絡作者、電子郵件：tcede@wufeng.tari.gov.tw)

冬瓜 (*Benincasa hispida* Cogn.) 上發現一種新病害病徵，葉部褪綠、葉脈突起及褪色、葉面皺縮、甚至捲葉之現象，使得株生長勢衰弱。採自彰化縣北斗鎮之冬瓜疑似病罹病株 (Wg1)，經瓜類作物的八種病毒血清検測，包括瓜類蚜媒黃化病毒 (*Cucurbit aphid-borne yellows virus, CAYBYV*)，胡瓜綠斑嵌紋病毒 (*Cucumber green mottle mosaic virus, CGMMV*)，胡瓜嵌紋病毒 (*Cucumber mosaic virus, CMV*)，甜瓜脈緣嵌紋病毒 (*Melon vein-banding mosaic virus, MVbMV*)，木瓜鉤點病毒-西瓜系統 (*Papaya ringspot virus W type, PRSV-W*)，番茄斑點萎蔫病毒 (*Tomato spotted wilt virus, TSWV*)，西瓜銀斑病毒 (*Watermelon silver mottle virus, WSMoV*)，及矮瓜黃花嵌紋病毒 (*Zucchini yellow mosaic virus, ZYMV*)，均呈陰性反應。針對 begomovirus外鞘蛋白之高度保留區域核酸序列，經比對設計引子對，以PCR増検之。經由噬菌感受細胞的轉形作用與核酸酶切，獲得1116個核苷酸，分為AV2 (126個核苷酸)、CP (771個核苷酸)及AC3 (219個核苷酸)。經與其他 begomoviruses 比對，得知 Wg1 病毒分離株與 *Squash leaf curl Philippines virus* 最有親緣關係，其核酸相當度在AV1, CP, 和 AC3 基因分別為97.6, 97.7, 和93.8 %。利用載體 pET21d (+) 火帶SqLV-Wg1 的 CP 基因，在 *Escherichia coli* BL21 (DE3) 中表現病毒鞘蛋白，並用火療滅活西蘭白兔製備專一性的抗血清，供進一步診斷鑑定用。本文為台灣瓜類感染 *Squash leaf curl virus* 的首次紀錄。