First Detection and Characterization of Chrysanthemum virus B infecting Chrysanthemum in Thailand

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Research Article

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Abstract

Chrysanthemum is one of the important ornamental plants in worldwide due to its high economic and cultural values. Chrysanthemum leaves showed mosaic, ringspot, yellowing and mild mottle symptoms were observed and collected from cultivation areas in northern Thailand and used for detection of important viruses infecting chrysanthemum. *Chrysanthemum virus B* (CVB) was detected by reverse transcription polymerase chain reaction (RT-PCR) from samples showing yellowing and mild mottle symptoms. Sequences of the coat protein (CP) gene of two CVB isolates found in this study were sequenced and shared 93.15% homology with other CVB isolates from different countries deposited in GenBank. Biological indexing of these CVB found that they induced both local and systemic symptoms in tobacco plants while petunia displayed a systemic symptom. The particles of CVB were observed under transmission electron microscope (TEM), prepared by dip preparation and negative staining methods, showing slightly flexuous rod-shaped virions approximately 600–650 nm in length. To our knowledge, this is the first detection and study on molecular and biological characteristics of CVB infecting chrysanthemum in Thailand.

Background

Chrysanthemum (*Chrysanthemum morifolium*) is a commercially important ornamental plant in worldwide which is grown as cut flowers and pot plants. Traditionally, chrysanthemums are mainly propagated through cutting, which is highly conducive to the accumulation and spread of virus and viroid diseases [7].

Virus diseases are main limiting factors for chrysanthemum cultivation. Many viruses are known to infect chrysanthemum including *Chrysanthemum virus B* (CVB) [14], *Cucumber mosaic virus* (CMV) [19], *Chrysanthemum stem necrosis virus* (CSNV) [19], *Impatients necrotic spot virus* (INSV) [4], *Tobacco mosaic virus* (TMV) [24], *Tomato aspermy virus* (TAV) [11], *Tomato spotted wilt virus* (TSWV) [8] and *Zucchini yellow mosaic virus* (ZYMV) [10]. To eradicate virus contamination in propagated cuttings, meristem tissue culture technique was used for production of virus-free chrysanthemum plantlets [15].

Among of them, CVB was found to be the most frequently chrysanthemum-infecting virus. CVB, formerly known as *Chrysanthemum mild mosaic virus*, is a member of the Genus *Carlavirus* in the Family *Betaflexiviridae* [16]. CVB has slightly flexuous rod-like particles with 685 nm in length and 12 nm in width. CVB has no protein envelope containing a single linear, positive-sense, single-stranded RNA genome with 8000–9000 nucleotides, excluding the poly(A) tail, containing six open reading frames [17]. CVB is globally distributed where chrysanthemums are grown and most commercial cultivars are usually infected by CVB without showing visible symptoms, but some cultivars showed mild mosaic, vein clearing or necrotic streak symptoms on leaves and the flowers, sometimes, are malformed [3][6][12]. The transmission of this virus occurs by some aphids consisting *Myzus persicae* and *Aphis gossypii* as a non-persistent manner and is also sap-transmissible [13].

CVB has been reported in many countries of Asia including India [22], Japan [23], Taiwan [6] and China [24]. In Thailand, chrysanthemums have been grown for decades, but the detection of viruses has not been reported. Therefore, the objectives of this study were to detect and identify CVB infecting chrysanthemum along with the description of molecular, biological and structural characteristics of the virus.

Surveys were conducted in chrysanthemum plantations in northern Thailand consisting Chiang Mai and Chiang Rai Provinces during 2019-2020. Chrysanthemum leaves from different cultivars showing virus-like disease, such as mild mottling, vein clearing, and yellowing were collected for detection of CVB. The percentage of disease incidence (PDI) was calculated as described by [1].

Total RNA was extracted from 0.1 g chrysanthemum leaves by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instruction. The reverse transcription reaction was performed by ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) following the protocol provided by the manufacturer. The primer specific to the coat protein (CP) gene of CVB include forward primer (5'-AGTCACAA TGCCTCCCAAAC-3') and reverse primer (5'-CATACCTTTCTTAGAGT GCTATGCT-3') [2] was used for PCR amplification which was performed by using EconoTaq® PLUS & PLUS GREEN 2X Master Mixes (Lucigen, USA) in the DNA Engine® Peltier Thermal Cycler PTC-200 (Bio-Rad, USA). The PCR reaction was performed as follow: 1 cycle of initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 54°C for 45s, extension at 72°C for 45s and 1 cycle of final extension at 72°C for 7 min. The PCR products were visualized on 1.5% agarose gel electrophoresis stained with RedSafe™ Nucleic Acid Staining Solution (iNiTron, Korea) in the MIL-DUT48 Blue light transilluminator (MIULAB instruments, China). The size of PCR products was compared to GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, USA).

Total of 4 chrysanthemum plantation areas were surveyed and 110 samples of chrysanthemum leaf were collected. From the detection of total 110 samples, PCR products of 621 bp specific to the CP gene of CVB were detected from 2 samples (BW-54 and HL4-70; the infection rate was 1.81%) showing yellowing and mild mosaic symptoms, both samples were collected from Chiang Mai Province (Fig. 1A-B). Symptoms observed from positive samples were accordance with typical symptoms induced by CVB consisting of mosaic, mottling and vein clearing [3][22]. In Taiwan, most of CVB-infected chrysanthemum showed symptomless, but only a small percentage of plants exhibiting mild mottling symptoms on their leaves [6].

CVB was found most frequently chrysanthemum-infecting viruses followed by CMV, *Tomato aspermy virus* (TAV), members of *Tospovirus* and *Potyvirus*, respectively [22]. In India, CVB was generally found to be double infections with CMV and showed the characteristics symptoms [21]. In this study, we detected those viruses by RT-PCR, but the detections showed negative results (data not show). Therefore, this assumed that the CVB infection in chrysanthemum found in this study was a single infection.

Nucleotide sequences of partial CP gene were directly analyzed using fluorescent dye-terminator sequencing on ABI Prism™ 3730xl DNA sequencers (Applied Biosystems, Foster City, CA). The obtained sequences were aligned by ClustalW performed in DNAMAN Sequence Analysis Software (Lynnon Corporation, Vaudreuil, Quebec, Canada) and deposited in GenBank. The phylogenetic tree was constructed based on the Maximum Likelihood (ML) method implemented in the MEGA X [5].

The partial CP gene sequences of CVB-BW-54 (accession no. MW460254) and CVB-HL4-70 (accession no. MW460255) were 99.87% homology compared to each other and were 93.15% homology compared to isolates of India (accession no. AM039441.1 and AJ812569.1), China (accession no. JQ904593.1 and JQ904595.1), Russia (accession no. MH678701.1 and MH678703.1), Korea (accession no. LC010240.1), Japan (accession no. AB245142.1) and Poland (accession no. KJ489421.1). The phylogenetic tree showed that CVB-BW-54 (accession no. MW460254) and CVB-HL4-70 (accession no. MW460255) clustered into CVB clade and were closely clustered with isolates from India and Russia, but far apart from isolates of Japan and Poland (Fig. 2). Moreover, CVB isolates in this study were separated from other members of the genus *Carlavirus* included *Carnation latent virus* (CLV) (accession no. X52627.1), *Shallot latent virus* (SLV) (accession no. GU355922.1), *Lily symptomless virus* (LSV) (accession no. D43801.1) and *Hop latent virus* (HpLV) (accession no. EF394783.1) (Fig. 2).

To study the pathogenicity of CVB on indicator plants, the virus was extracted from infected chrysanthemum leaves, positive to RT-PCR detection, using 0.1 M phosphate buffer (pH 7.0) and then was mechanically inoculated to *Chenopodium quinoa* for single virus isolation and subsequently inoculated to 4 species of tobacco including *Nicotiana tabacum* cv. Xanthi, *N. tabacum* cv. Samsun, *N. benthamiana, N. glutinosa* and petunia (*Petunia hybrida*) for virus multiplication and maintenance. RT-PCR was used for confirmation of the virus infection.

At 10 day-post inoculation (dpi), *C. quinoa* produced chlorotic spots on inoculated leaves which were found from both isolates. CVB isolate BW-54 and HL4-70 induced local necrotic spots on inoculated leaves of *N. glutinosa* and *N. tabacum* cv. *Samsun* at 3 dpi and subsequently developed to necrosis. Systemic infections were observed on *N. benthamiana* and *N. tabacum* cv. Xanthi at 7-10 dpi with mosaic and malformation of newly developed young leaves. Petunia showed systemic mosaic with malformation symptoms on upper leaves at 21 dpi. These 2 two isolates of CVB had the similar biological properties. The present of CVB was detected by RT-PCR from systemically infected leaves and the amplicons of 621 bp of CVB CP gene were visualized (data not shown).

As CVB was difficult for mechanical inoculation to chrysanthemum [3] then this study demonstrated that *N. benthamiana*, *N. tabacum* cv. *Xanthi* and *P. hybrida* were suitable for CVB propagation, due to systemic symptoms, which were similar to [9] and [17] reporting that CVB had quite narrow host range which was apparently restricted to chrysanthemum, a few *Nicotiana* species and petunia. However, CVB also had *Vicia faba* (Family *Leguminosae*) and tetragonia (Family *Aizoaceae*) as systemic hosts [9].

Particles of CVB from inoculated tobacco leaves were observed under TEM JEM 2010 (JOEL, USA). Leaves were prepared by dip-preparation method and then negatively stained [18] with minor

modifications. Briefly, leaves were ground with 0.1 M phosphate buffer in the ration 1:1 (w/v), centrifuged at 12,000 rpm for 5 min and the clear supernatant was collected. Dip preparation was conducted by dropping the supernatant on foamvar-coated carbon grid (300 mesh) (Sigma-Aldrich, USA), fixed by using 1% glutaraldehyde and negatively stained with 2% uranyl acetate.

Transmission electron micrograph of CVB-BW-54 and CVB-HL4-70 particles from inoculated tobacco leaves showed slightly flexuous rod-shaped particles approximately 650-680 nm in length (Fig. 3). The observed virions were similar to CVB from India which had typical particles of *Carlavirus* with about 650-680 nm of flexuous rod particles [22].

In conclusion, the CVB infection in chrysanthemum of Thailand was newly found. Two isolates of CVB were isolated and studied on molecular and biological characteristics. Comparison of partial sequences of CP gene of these isolates found that they shared 93.15% identity with other CVB isolates from GenBank. Biological indexing showed that some species tobacco and petunia could be used as propagative host plants. Like *Carlavirus*, CVB has slightly flexuous rod-shaped particles observing under TEM. To our knowledge, this is the first report of CVB detecting from chrysanthemum in Thailand along with the description of molecular and biological characteristics.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals per- formed by any of the authors.

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Figures

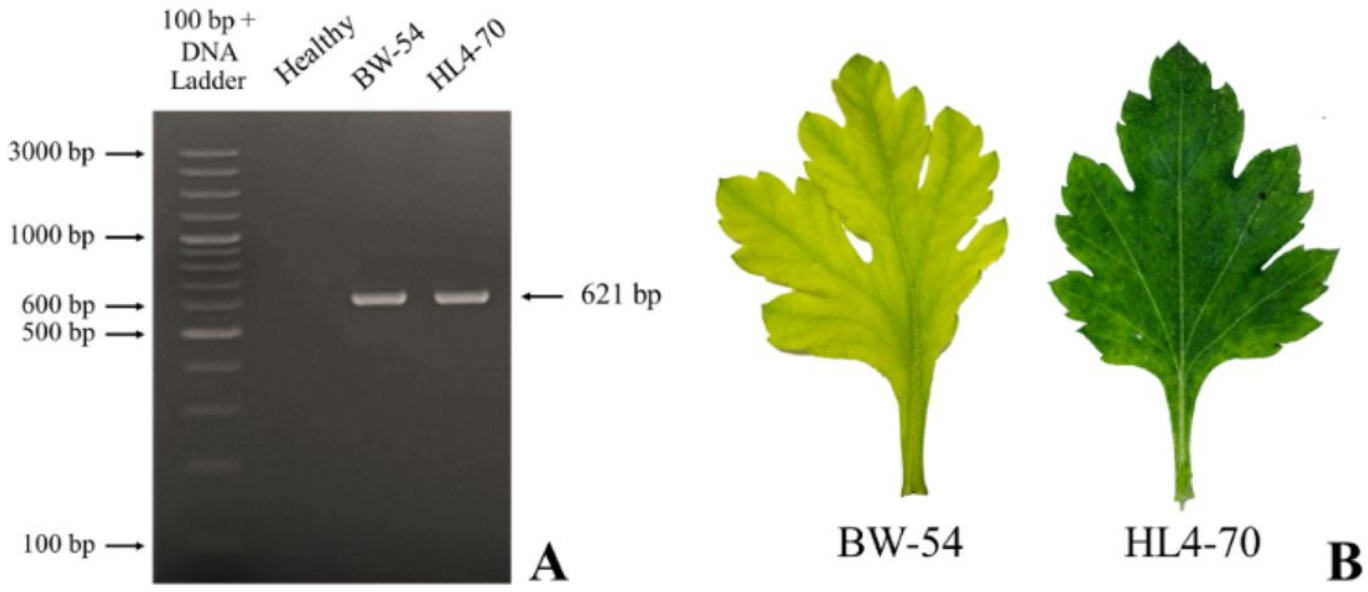

Figure 1

RT-PCR detection of Chrysanthemum virus B (CVB). (A) Amplicons of 621 bp of CVB coat protein (CP) gene and (B) leaves of chrysanthemum BW-54 and HL4-70 showed yellowing symptom and mild mottling, respectively, which were positive to CVB detection.

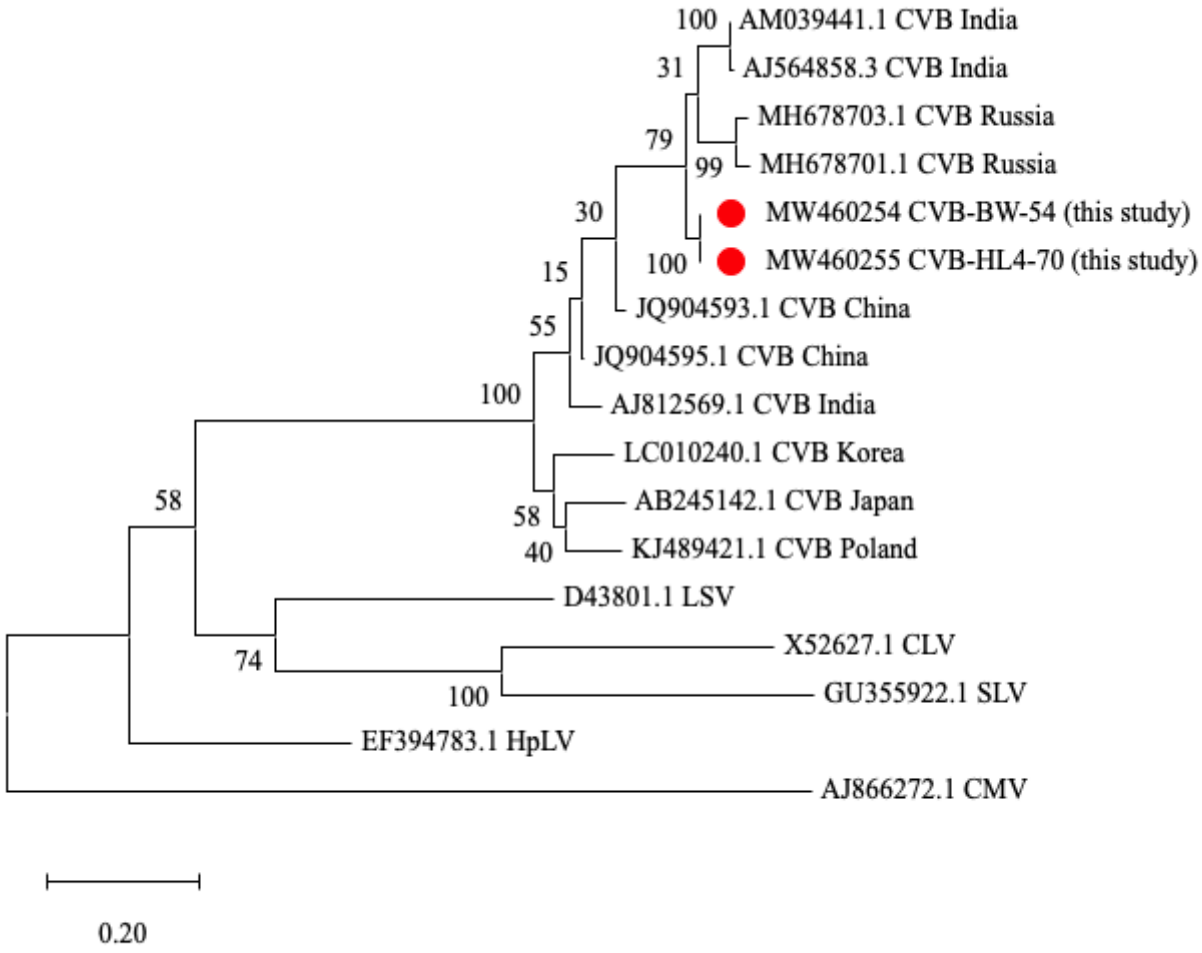

Figure 2

Phylogenetic relationship of CVB-BW-54 and CVB-HL4-70 (indicated by red circles) and members of the genus Carlavirus based on nucleotide sequences of partial coat protein (CP) gene. Cucumber mosaic virus (CMV), genus Cucumovirus, was used as outgroup. Multiple sequence alignments and construction of the phylogenetic tree were generated by ClustalW by the Maximum Likelihood (ML) method with 1,000 replicates of bootstrap values performed by MEGA X program.

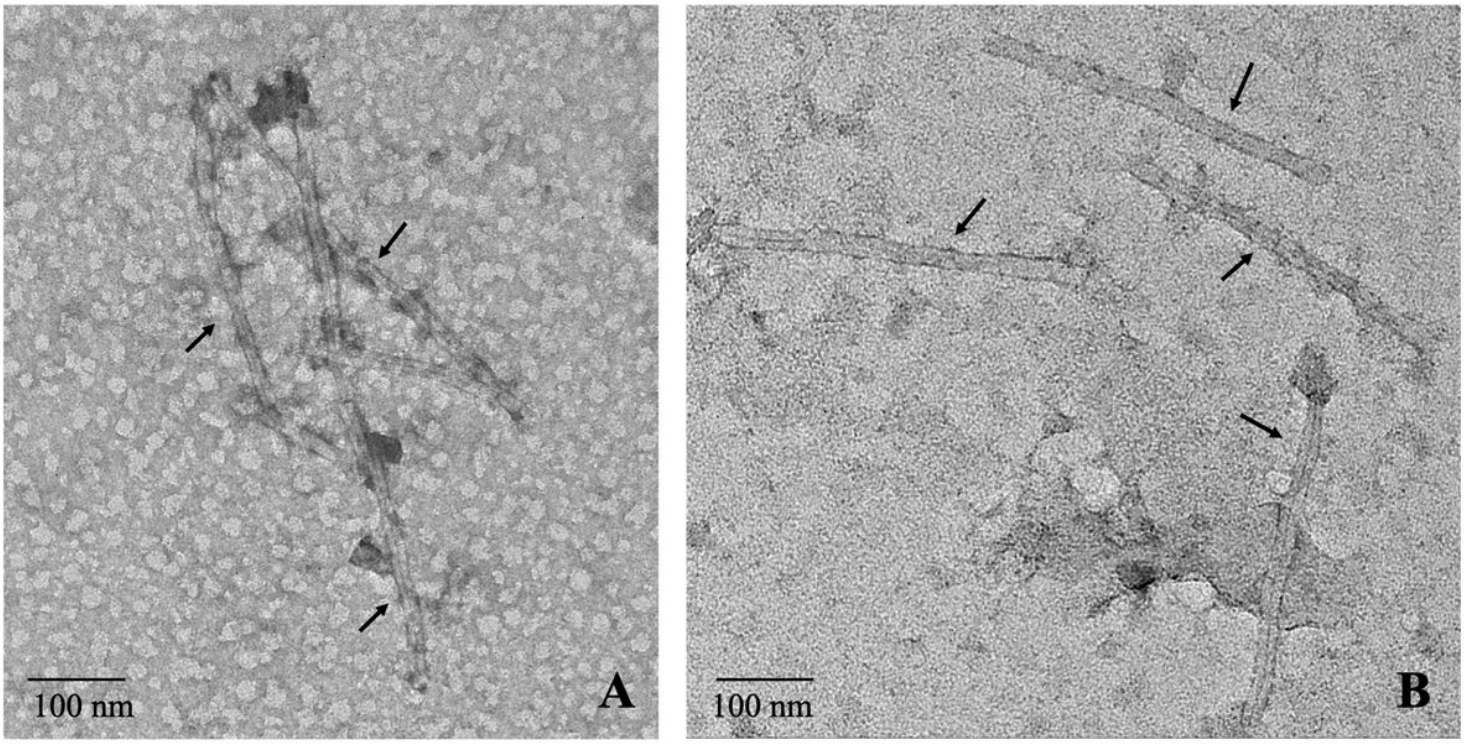

Figure 3

Transmission electron micrograph of negatively stained CVB showed slightly flexuous rod-shaped particles (indicated by arrows) (A) CVB-BW-54 and (B) CVB-HL4-70.