

Identification of papaya ringspot virus W type infecting cucurbits in Ho Chi Minh City, Vietnam

Bich T. N. Tran^{1*}, Nghi T. T. Nguyen¹, Nam N. Doan¹, Nien C. Nguyen², & Trang T. H. Nguyen³

¹Department of Plant Protection, Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam

²Department of Plant Genetic Breeding, Faculty of Agronomy, Nong Lam University,
Ho Chi Minh City, Vietnam

³Department of Industrial Crops and Medicinal Plants, Faculty of Agronomy, Nong Lam University,
Ho Chi Minh City, Vietnam

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*Corresponding author

Tran Thi Ngoc Bich

Email:

ttnbich@hcmuaf.edu.vn

ABSTRACT

Papaya ringspot virus W (PRSV W) is a common aphid-transmitted potyvirus, seriously affecting cucurbit production worldwide. This study conducted field surveys to collect PRSV W infecting cucurbit crops in Cu Chi and Hoc Mon districts, two major vegetable-growing areas of Ho Chi Minh City. A total of 48 samples representing sponge gourd, cucumber, bottle gourd, wax gourd, bitter gourd, and pumpkin were collected and tested by indirect ELISA-based serological assay, which detected PRSV in four samples. The indirect ELISA-positive cucurbit samples were then subject to bioassay to identify the type of PRSV, followed by reverse transcription polymerase chain reaction (RT-PCR) confirmation for the PRSV W type. Among all the symptomatic cucurbit samples collected and tested in this study, the highest incidence (27.3%) of PRSV W was recorded on the sponge gourd samples. The infected leaves' extracted total RNA was analyzed by RT-PCR with primer pairs SMK 8701(F)/3'UTR(R), and the PCR product was sequenced. The results revealed that the complete CP genome sequence of isolate PRSV W isolate HocMon (PRSV W-HM) was most closely related (polyprotein nt and aa identities of 93.3 and 95.1%, respectively) to the China isolate of PRSV W (GenBank KX6558741). Briefly, this study provides important insights for the accurate early diagnosis of PRSV W infection and developing effective disease control strategies to limit the spread of this virus isolate.

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1. Introduction

Vegetable crops are essential for human nutrition, and cucurbits are the largest group of tropical vegetables (Roy & Chakrabarti, 2003). Many economically important crops worldwide belong to the family Cucurbitaceae, including cucumber, watermelon, pumpkin, and watermelon. Diseases caused by viruses on cucurbits result in significant yield loss (Romay et al., 2014). In Ho Chi Minh City, Vietnam, the major vegetable areas are located in the Binh Chanh, Hoc Mon, and Cu Chi districts. The total vegetable cultivation area in Ho Chi Minh City is 3,200 ha, concentrated in the districts of Cu Chi 1,900 ha, Binh Chanh 570 ha, and Hoc Mon 390 ha (HSDCPPP, 2024).

Papaya ringspot virus, scientific name PRSV *Potyvirus papayanuli*, is a member of *Potyvirus*, the largest genus of the family Potyviridae, with over 230 virus species in 12 genera, accounting for approximately 30% of all plant viruses (Petrov et al., 2023). The viruses in the genus *Potyvirus* have single-stranded, positive-sense RNA genomes with a poly-A tail at the 3' end (Purcifull, 1984; Tripathi et al., 2008) and non-enveloped, flexuous, and filamentous particles, measuring 11 - 20 nm in diameter and 650 to 950 nm in length (Inoue-Nagata et al., 2022). The PRSV is divided into two types: W type (PRSV W) infects cucurbit (*Cucurbitaceae*) and quinoa (*Chenopodium spp.*) plants but not papaya (Rezende & Pacheco, 1998), and P type (PRSV P) infects plants of papaya (*Carica papaya* L.), cucurbits, and quinoa (Purcifull, 1984).

In Vietnam, PRSV W is one of the viruses that cause serious damage to cucurbit plants, accounting for 47% of the total samples investigated in Vietnam (Revill et al., 2004). The PRSV P is seriously limited in papaya growing in Vietnam due to the devastating effects (Tran

et al., 2023a). It is necessary to determine the type of virus because the strategy to control each type of virus is different. PRSV W is one of the most prevalent aphid-borne viruses affecting cucurbits, causing severe losses in a wide range of cucurbit crops globally (Gadhav et al., 2020; Pozzi et al., 2020). Symptoms of this PRSV W viral disease include mosaic symptoms and deformation in leaves and fruits (Krubphachaya et al., 2007). In North Vietnam, most PRSV W isolates were collected from various cucurbit hosts, including pumpkin, waxy gourd, bottle gourd, cucumber, sponge gourd, and zucchini squash (Bateson et al., 2002; Revill et al., 2004).

In this study, we surveyed PRSV W on cucurbit plants in some big vegetable areas in Ho Chi Minh City, Vietnam. A field survey, enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), and sequencing of the coat protein (CP) gene region of cucurbit samples collected from different vegetable areas of Ho Chi Minh City revealed that PRSV W isolate HocMon (PRSV W-HM) was collected from sponge gourd and wax ground. Our findings contribute to accurately diagnosing PRSV W infection in the early stage and to developing effective control strategies.

2. Materials and Methods

2.1. Survey of papaya ringspot virus W type disease

The field surveys were conducted from November 2023 to January 2024 in some big vegetable areas in Ho Chi Minh City, including Hoc Mon and Cu Chi districts. The 48 samples were collected from different locations in Hoc Mon district (Xuan Thoi Thuong, Xuan Thoi Son and Tan Thoi Nhi Communes) and Cu Chi district (Pham Van Coi, An Nhon Tay and Nhuan Duc communes).

The plants showing virus symptoms of PRSV W, including mosaic, malformation, blistering of leaves, mottled, and malformations on fruits (Guner et al., 2008; Lecoq & Desbiez, 2012) were collected from cucurbit namely cucumber, pumpkin, and gourd in cropping areas and backyards of farmers. As plants can be infected with other viruses in the field, samples were collected, stored in a cool box, and transported to the Department of Plant Protection, Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam for further diagnosis tests.

2.2. Serological virus detection by ELISA

The presence of PRSV W virus in field-collection samples was detected by serological method using indirect enzyme-link immunosorbent assay (ELISA) using the polyclonal antiserum to PRSV CP (Yeh et al., 1984). Mock-inoculated plants with buffer and plants inoculated with severe PRSV W were included as negative and positive controls, respectively. The indirect ELISA method followed the procedure (Yeh et al., 1984) used for PRSV detection with some modifications. Briefly, leaf tissue was extracted at 1:50 (w/v) in coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , 3.1 mM NaN_3 , pH 9.6) and 200 μL of ground tracts were added into each well of the microtiter plate. After incubating at 37°C for 1 h, the plate was washed 3 times (3 min/time) with 1X PBST (137 mM NaCl , 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 2.7 mM KCl , 3.1 mM NaN_3 , Tween-20 0.025%, pH 7.4). Antigen-precoated wells were loaded with 200 μL polyclonal antibody diluted with conjugated buffer (PBST 100%, 18 mM PVP, 30.1 mM BSA) before incubating at 37°C for 1 h. The plate was washed with 1X PBST 3 times and incubated with goat-anti-rabbit IgG diluted to 1:5000 with conjugated buffer (200 μL /well) at 37°C for 1 h. After repeating the plate washing step 3 times with PBST, add 150

μL aliquots of freshly prepared substrate buffer (Diethanolamine 9.7%, 3.1 mM NaN_3) to per well (1 mg/mL p-nitrophenyl phosphate in substrate buffer and incubated at room temperature for about 20 min. The results were measured at 405 nm with an ELISA reader (Alta ELISA reader adx-110, Athenese-Dx Private Limited, CTK Biotech Inc, USA).

2.3. Pathotyping through bio-assay

After confirmation of the infection by ELISA, the samples infected with PRSV were extracted mechanically with phosphate buffer (pH 7.0) to *Chenopodium quinoa* Willd until local lesions appeared. Continue inoculating to the next quinoa plant by cutting a local lesion of ground with buffer and repeating this step 3 times until the lesion is clear to purify the virus. The purified virus was then transferred to horn melon (*Cucumis metuliferus*) in the greenhouse for symptom observation.

The bio-assay experiment was performed through mechanical sap transmission of the purified virus to the differential hosts. These are muskmelon (*Cucumis melo*), horn melon (*C. metuliferus*), cucumber (*C. sativus*), watermelon (*C. lanatus*), bottle gourd (*Lagenaria siceraria*), bitter gourd (*Momordica charantia*), quinoa (*Chenopodium quinoa*), and papaya (*Carica papaya*) with 10 plants for each species. A 0.1 g leaf tissue sample was homogenized with the cold 0.01 M potassium phosphate buffer of pH 7.0. Using a sterilized cotton swab, the homogenates were mechanically introduced to the two-leaf plants dusted with carborundum at the two-true-leaf stage. About 2 min after the sap was applied, the inoculated leaves were gently washed with clean water. The inoculated plants were kept in the greenhouse and recorded the symptom expression.

2.4. RT-PCR based confirmation of PRSV pathotyping

After confirmation of the infection by ELISA in section 2.2 and pathotyping through the bio-assay in section 2.3, the samples infected with PRSV were extracted mechanically with phosphate buffer (pH 7.0) to *Chenopodium quinoa* Willd until local lesions appeared. Continue inoculating to the next quinoa plant by cutting a local lesion of ground with buffer and repeating this step 3 times until the lesion is clear to purify the virus. The purified virus was then transferred to horn melon (*Cucumis metuliferus*) in the greenhouse for symptom observation. Two weeks after inoculation, leaves of horn melon were inoculated showing system symptoms.

The leaf tissues 0.1 g from the infected plant were used for total RNA extraction by TRIsure™ reagent (Meridian Bioscience, USA) following the manufacturer's protocol. 600 ng total RNA and oligo (dT)₁₈ primer mix were used for complementary DNA (cDNA) synthesis by Tetro cDNA Synthesis Kit (Meridian Bioscience, USA) following the manufacturer's protocol. For polymerase chain reaction (PCR) assay, 2 microliters of cDNA as template and the primer pair SMK 8701 (F) TCTCTGTATCGCCATTCACCCGGATC and 3'UTR (R) CTCTCATTCTAAGAGGCTCGAATAGC (Tran et al., 2022) were used to amplify the fragment containing the entire coat protein (CP). The cycling parameters were as follows: initial denaturation at 95°C for 1 min, 35 cycles of denaturation at 94°C for 20 sec, incubation at 54°C for 20 sec, and extension at 72°C for 1 min 45 sec, followed by a final extension step of 72°C for 5 min (Lorenz, 2012). The amplification products were visualized by electrophoresis on a 1% agarose gel. The RT-PCR product was further analyzed by electrophoresis using 1% agarose gel and recorded by UV gel Vilber Lourmat

system (Vilber, France) to observe the CP gene amplification. The PCR product was purified by DNA clean kit (GeneMark, Gmbiolab, Taichung, Taiwan) following the manufacturer's protocol before sending to the Nam Khoa Trading and Service Company Limited for DNA sequencing.

2.5. Phylogenetic analysis of PRSV W CP sequences

To analyze the relationships of the PRSV W-HM isolate with other PRSV W isolates, the coat protein (CP) sequence of PRSV W-HM was compared with the CP of other PRSV W from the NCBI GenBank database. The nucleotide and amino acid sequences were compared by the vector NTI program (Vector NTI Advance 11.5.1, Invitrogen, Life Technologies Corporation, CA). The CP sequences were further aligned by clustal W and phylogenetic trees were constructed by MEGA11 with the maximum likelihood algorithm from DNA sequences with a bootstrap value set at 1,000 replicates.

3. Results

3.1. Sample collection and symptomatology

The PRSV W is known to induce mosaic, malformation, blistering of leaves, mottled, and malformation on fruits in different cucurbit hosts (Lecoq & Desbirz, 2012; Tran et al., 2023b). Based on these symptoms, cucurbit samples were collected from different districts of Ho Chi Minh City. In this field investigation, a range of symptoms, including vein banding, malformation, curling, distorting, and crinkling of a leaf, are also revealed. The location of the field investigation and sample collection of PRSV isolates in this study are shown in Figure 1A. The viral-infecting-like symptoms including severe mosaic with malformation, mottling, and distortion were found on sponge and wax gourd (Figure 1B). Cucumber mosaic virus (CMV) and cucumber green mottle

mosaic virus (CGMMV) have been reported with similar symptoms (Wang et al., 2020; Kehoe et al., 2022). As a result, 4 out of 48 samples were detected as positive for W type by indirect-ELISA, plant-indicator, RT-PCR, and sequencing.

3.2. Indirect-ELISA diagnosis

PRSV detection with the indirect-ELISA method on 48 samples with virus-like symptoms were collected in big vegetable areas in Ho Chi Minh City. Out of a total of 48 samples tested by indirect-ELISA, 4 samples were found associated with PRSV W. Among them, 3 positive samples were identified from 11 sponge gourd samples collected, representing 27.3%. Samples collected from each location were loaded with

per well of ELISA plate coated with specific antibodies. Healthy tissue (mock) and a PRSV W-infected sample were also used as controls. The absorbance values OD405 nm of collected samples, including 2 samples of sponge gourd (*L. aegyptiaca*) collected in Nhuan Duc Commune (Cu Chi District), one sample in Tan Thoi Nhi Commune (Hoc Mon District), a sample of wax gourd (*B. hispida*) collected in Xuan Thoi Thuong Commune (Hoc Mon District), showed positive results with PRSV CP antibody (Figures 1C and 1D). However, relying on symptoms to collect samples was not very effective because only 4 sample species tested positive for ELISA out of a total of 48 collected samples, all of which had similar symptoms of PRSV infection.

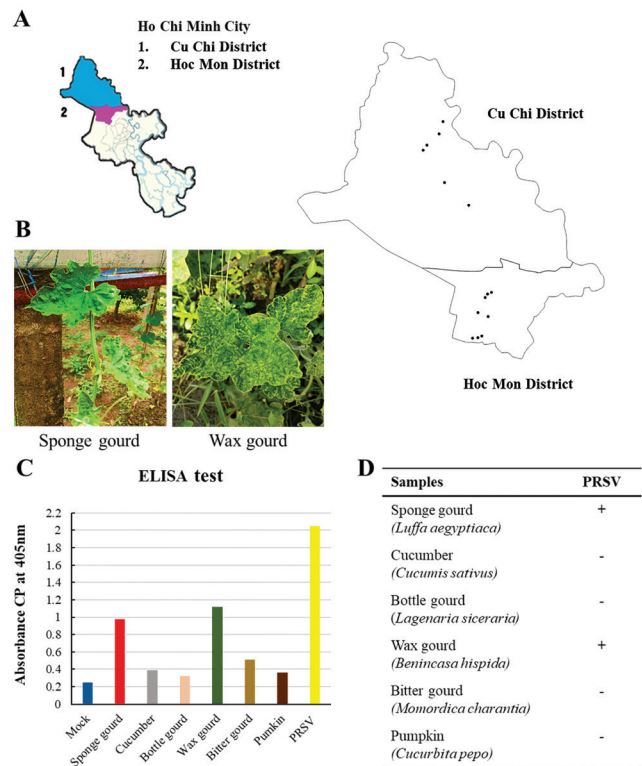


Figure 1. Symptomatology and indirect, antigen-coated ELISA detection of PRSV isolates in field-collected tissue samples. A: Location of the filed investigation and sample collection of PRSV isolate in this study. B: Foliar symptoms of two samples were positive with PRSV W. C: The absorbance values of samples were detected by ELISA using antiserum against PRSV CP. D: Results determined the presence of severe virus isolates PRSV W in 6 plant species. PRSV W: Papaya ringspot virus W, PRSV CP: Papaya ringspot virus coat protein.

3.3. Symptoms of some common cucurbits inoculated with PRSV W-HM isolate

The pathotyping studies were conducted through bio-assay by inoculating the sap from an indirect-ELISA positive cucurbits sample. All samples induced virus-infected-like local symptoms on the test plants of *Chenopodium quinoa*. Plants of muskmelon, horn melon, cucumber, watermelon, bitter gourd, and bottle gourd, inoculated with this virus isolate showed mosaic, leaf distortion, and blistering symptoms, whereas papaya plants were immune and did not show any symptoms (Figure 2). Specifically, mosaic, puckering, and blistering symptoms were observed on muskmelon leaves.

On horn melon and cucumber, PRSV W causes mosaic, malformation on leaves, and curling on watermelon was observed. On bitter gourd and bottle gourd, PRSV W causes malformation, and curling, and reduces the size of leaves.

Plants of the common cucurbits inoculated with this virus isolate showed mosaic, leaf distortion, and blistering symptoms, whereas papaya plants were immune and did not show any symptoms. These results concluded that the PRSV isolate obtained from survey samples is PRSV W. Then, the RNAs of disease plants were extracted, and PRSV CP genes were sequenced to confirm this result again.

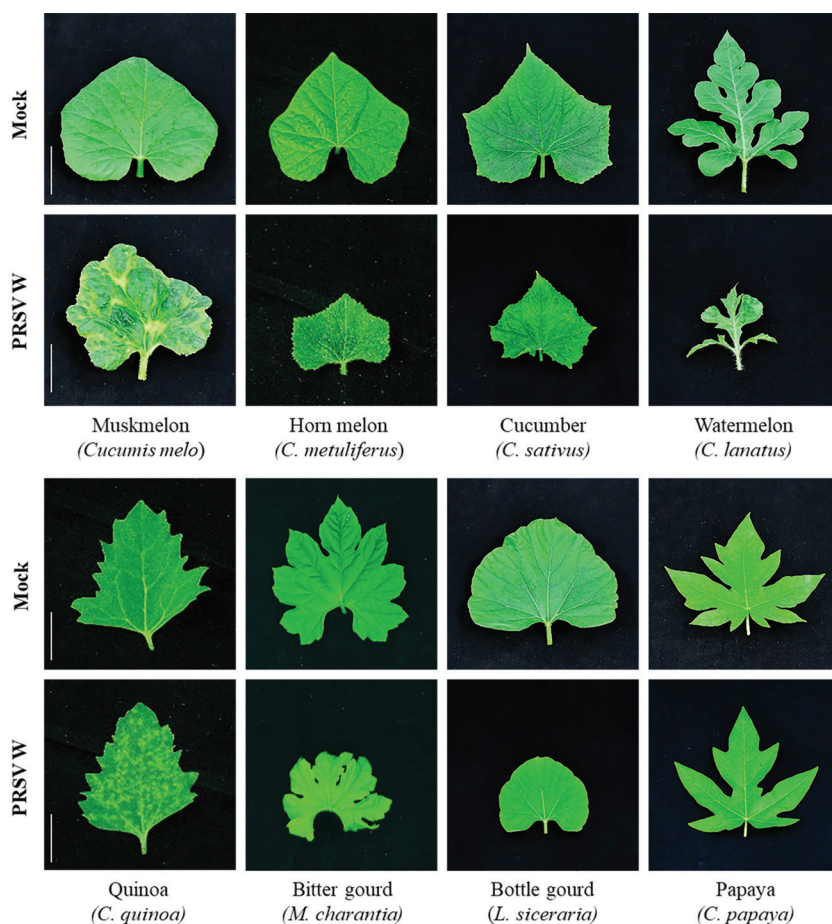


Figure 2. Different types of symptoms developed on local lesion host *Chenopodium quinoa* (15 dpi), and systemic symptoms developed in muskmelon, horn melon, cucumber, watermelon, bitter gourd, and bottle gourd (30 dpi) after inoculation with PRSV W. PRSV W: Papaya ringspot virus W.

3.4. RT-PCR based confirmation of pathotyping

After isolating on quinoa three times, the bio-assay positive plant sample was inoculated on systemic horn melon (*C. metuliferus*). The infected plant developed severe symptoms indicating successful infection of PRSV W. This plant was also confirmed by RT-PCR with specific primers SMK 8701 (F) and 3'UTR (R) to amplify the CP fragment of PRSV W (Figure 3A).

The plants that showed positive in the test plants, were also found positive in RT-PCR assay (Figure 3B). A cDNA fragment of 1650 bp amplified by 8701 (F) and 3'UTR (R) primers from infected plant tissue indicated the infection of severe PRSV. These results confirmed again that the sample that could induce the symptoms on cucurbits and not show any symptoms on papaya plants was associated with the PRSV W type. In addition, the RT-PCR product was further sequenced to identify the virus isolation.

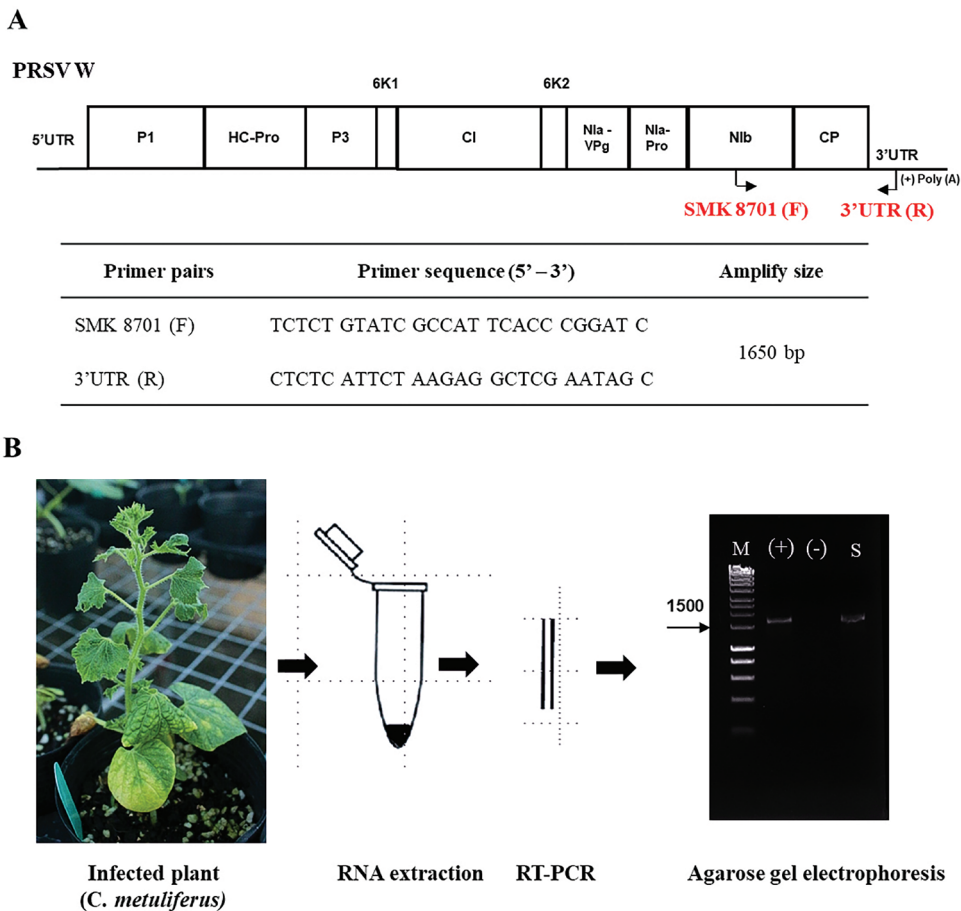


Figure 3. RT-PCR determination of Papaya ringspot virus W causing on cucurbit crops in Ho Chi Minh City. A: Schematic representation of the PRSV W genomes. The arrow and red number indicate the RT-PCR products of 1650 bp were amplified with specific primers to amplify the fragment. UTR: Untranslated region, P1: Protease, HC-Pro: Helper Component protease, CI: Cylindrical Inclusion, VPg: Viral Protein genome linked, Nla-Pro: Nuclear Inclusion a protease, Nlb: Nuclear Inclusion b and CP: Coat Protein. B: The graphical illustration of RT-PCR steps, read from the infected plant (left) to the agarose gel electrophoresis of RT-PCR products for the detection of PRSV W in the leaf (right). (+): positive control, (-): negative control, (S): Testing sample, M: Marker.

3.5. Phylogenetic analysis of PRSV W-HM

To analyze the relationship of PRSV W-HM with other potyviruses from the NCBI GenBank database, a phylogenetic tree generated from the CP sequence of PRSV W-HM isolate was compared to other PRSV W isolated from the NCBI. Our result showed that PRSV W-HM is closest to the China, Thai Lan, and Taiwan isolates (Table 1 and Figure 4A).

The maximum nucleotide identity of 93.3 and 92.9% were with the PRSV W isolates KX655874.1 and KX998707.1, both from China (Table 1). Additionally, the PRSV W-HM isolate had a nucleotide identity ranging from 93.2 and 92.7% when compared to the other PRSV isolates AY027810.2 from Taiwan, and AY01722.1 from Thailand. Those countries that are geographically near Vietnam. The PRSV W followed this isolate KX655872.1 reported from Australia and OM687252.1 from the USA, with an 89.6% nucleotide identity.

Table 1. Analysis of sequence variation in coat protein (CP) gene of PRSV W-HM isolate of this study with PRSV W isolated deposited in NCBI GenBank

GeneBank accession number	Nucleotide identities (PRSV W-HM)	Amino acid identities (PRSV W-HM)	Percentage identity	Coverage rate
KX655874.1 (China)	93.3%	95.1%	93.7%	100%
KX998707.1 (China)	92.9%	97.4%	93.6%	99%
AY010722.1 (Thailand)	92.7%	96.1%	93.4%	99%
AY027811.1 (Taiwan)	93.2%	96.4%	93.3%	100%
KX655872.1 (Australia)	89.6%	94.5%	90.5%	99%
OM687252.1 (USA)	89.6%	94.8%	90.8%	98%
DQ374152.1 (Brazil)	89.3%	92.2%	90.3%	99%
LC482263.1 (India)	86.3%	91.2%	88.7%	94%

PRSV W-HM: *Papaya ingspot virus W isolate Hoc Mon*.

When comparing the amino acid identities of PRSV W isolate to PRSV W from various regions of the world, the PRSV W-HM isolate showed the maximum amino acid identity of 97.4% with PRSV W isolate (KX998707.1) reported from China, 96.1% with PRSV W isolate (AY027810.2) from Taiwan, 95.5% with PRSV W

isolate (AY01722.1) from Thailand, and 95.1% with PRSV W isolate from China (KX655874.1). The PRSV W-HM isolate was classified as PRSV W because it was thought to be a variation of the described species according to phylogenetic and species demarcation criteria.

Together with a PRSV W-HM isolate, the whole nucleotide CP sequences of 24 PRSV W isolates that have been reported globally were used for phylogenetic analysis (Figure 4A). Four main lineages were identified from the PRSV isolates. The first lineage included 4 isolates from China, 6 isolates from the USA, 3 isolates from India, 3 isolates from Brazil, one isolate

from Australia, and one isolate from Cuba. A total of 18 isolates from these countries were included in this lineage. Four isolates from three nations that are geographically close to Vietnam, including two isolates from China, one isolate from Taiwan, and one isolate from Thailand are found in lineage two. There is only one isolate from India in the third and fourth lineages.

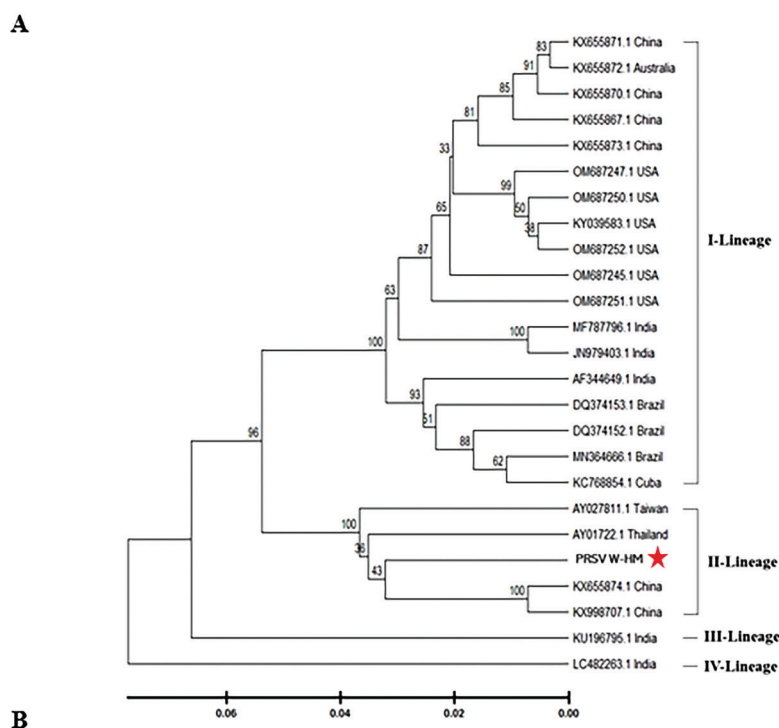


Figure 4. A: Phylogenetic tree illustrating the relationships between the PRSV W-HM isolate and other identified PRSV W isolates according to the coat protein sequences. B: The coat protein sequence of HM isolate (918 nucleotides). The accession numbers in NCBI GenBank and the names of countries are indicated. Next to the branches are percentages of duplicate trees where the related taxa clustered together in the bootstrap test (1,000 repetitions). PRSV W-HM: Papaya ringspot virus W isolate Hoc Mon.

4. Discussion

Cucurbit is an important crop widely cultivated in tropical regions, including Vietnam and other countries worldwide. There are three important viruses infecting cucurbits, including Cucumber mosaic virus (CMV), Zucchini yellow mosaic virus (ZYMV), papaya ringspot virus W (PRSV W). The new taxonomy, which has now been ratified by the ICTV, including CMV, ZYMV, PRSV W, and many other viruses has been converted to Latinized binomial names since 2023 (Zerbini et al., 2023). Among these viruses, PRSV W is one of the most widespread and damaging viruses that infect cucurbits, including many economically important crops worldwide, such as cucumber, watermelon, muskmelon, squash, and pumpkin (Chomicki et al., 2020). The large-scale cultivation and commercialization of cucurbit, especially muskmelon, have recently been successful in Ho Chi Minh City. However, recent studies have found that CMV infects cucumbers in Ho Chi Minh City, Tien Giang, and Lam Dong provinces (Nguyen et al., 2017), and the occurrence tendency increases gradually with time. Also, we investigated some big vegetable areas, and muskmelon commercial net houses, and found that muskmelon, bitter melon, and long bean crops were infected by three kinds of the virus, PRSV W, CMV, and ZYMV (data not shown). These results indicated that the viruses on cucurbits, specifically PRSV W, appear in Ho Chi Minh City and increased numbers in cultivated cucurbit crops.

To determine the sequence diversity within PRSV isolates from different geographical locations, coat protein (CP) nucleotide and amino acid sequences of PRSV isolates are analyzed (Portal et al., 2006; Singh et al., 2017). A comparison of coat protein sequences can reveal isolate variability. According to the symptom

characteristics of PRSV on some cucurbit species, the typical symptom caused by PRSV W is a severe mosaic, malformation, and blistering of leaves. This symptom was similar to those caused by the PRSV W Taiwanese isolate (Tran et al., 2023b). Thus, the CP sequence alignment between PRSV Taiwanese isolate and PRSV W-HM shows that the identities of nucleotides were 93.2%. This result implied that these isolates were relatively similar.

5. Conclusions

In this study, we collected cucurbit samples from November 2023 to January 2024, and analyzed samples based on symptoms, indirect-ELISA, RT-PCR, and phylogenetic tree. Our analysis and results confirm that the HM isolate is PRSV W type and highly similar to previously reported isolates from China (Accession number KX6558741 and KX998707.1), Thailand (AY010722.1), and Taiwan (AY027811.1). This is an important PRSV W-type infection report on cucurbits in Ho Chi Minh City.

Conflict of interest

The authors declare that they have no competing interests.

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