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Field Survey to Identify Mosaic Disease Viruses of *Cucurbita maxima* in Okinawa Prefecture, Japan

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Abstract

Squash (*Cucurbita maxima*) is an essential crop cultivated throughout Japan, and viral diseases such as mosaic disease cause a tremendous loss of squash yield in this region. Although surveying and detection of infectious viruses are essential for controlling viral diseases, currently, no in-depth field surveys have yet been conducted. Here, we conducted a field survey in virus-infected squash fields in Okinawa Prefecture, Japan. A total of 138 samples, including 131 from squash and 7 from weeds, were collected from Ishigaki Island, Miyako Island, and Okinawa Main Island. Nine identified viruses were then investigated by reverse transcription-polymerase chain reaction (RT-PCR). Most samples were found to be infected with zucchini yellow mosaic virus (ZYMV), and a few were infected with papaya ringspot virus (PRSV) or both ZYMV and PRSV. No other cucurbit-infecting viruses were detected among the samples. In addition, ZYMV and PRSV were detected in cucurbitaceous weeds grown near squash fields, suggesting that these weeds may act as an infection source for these viruses.

Discipline: Agricultural Environment
Additional key words: cucurbitaceous weeds, RT-PCR, squash

Introduction

Cucurbits are a widely cultivated group of crops that include squashes, which are widely grown both in Japan and around the world. The three main species of squashes are *Cucurbita maxima*, *C. moschata*, and *C. pepo* (Michael 1990), and *C. maxima* is the most widely grown species in Japan. Squashes are cultivated throughout Japan, including Hokkaido, Ibaraki, and Okinawa prefectures. In Okinawa Prefecture, squashes are mainly cultivated in winter. However, viral vectors, including aphids, thrips, and whiteflies, are present because of the warm climate, and therefore, they may cause concerns related to viral diseases. In Japan, 9 major viruses, namely, beet pseudo-yellows virus (BPYV), cucurbit chlorotic yellows virus (CCYV), cucumber green mottle mosaic virus (CGMMV), cucumber mosaic virus (CMV), kyuri green mottle mosaic virus (KGMMV), melon necrotic spot virus, melon yellow spot virus (MYSV), papaya ringspot virus (PRSV), watermelon mosaic virus (WMV), and zucchini yellow mosaic virus (ZYMV), have been identified on cucurbit plants (Fuji et al. 2022). Of these viruses, CMV, ZYMV, WMV, and PRSV have been detected in squashes (Phytopathological Society of Japan 2023). Those viruses cause severe damage and yield loss to the infected plants. Field surveys of infectious viruses are essential for controlling viral diseases because control strategies vary depending on the vector and virus of concern. However, the most current field surveys of infectious viruses affecting squashes were published several decades ago (Hokama et al. 1995, Nakayama et al. 1990, Yoshida & Iizuka 1987), and therefore, a new

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detailed survey of viruses in squashes should be conducted. Moreover, weeds are an essential reservoir for plant viruses, and in Japan, many perennial weeds that naturally grow around cucurbit crop fields belong to the family Cucurbitaceae (e.g., *Trichosanthes rostrata*, *T. cucumeroides*, and *Bryonopsis laciniosa*). Therefore, a survey of viruses present in nearby weeds is also required to control viral infection of commercial plantings. However, currently, few attempts have been made to detect viral pathogens in Cucurbitaceae weeds (Nakayama et al. 1990).

Diagnosing plant viruses is an essential task for crop protection. Polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) using sequence-specific primers for viral genes are key techniques involved in diagnosing plant viruses due to their highly sensitive and straightforward protocols (Roy et al. 2005). Previous studies have identified specific primers designed for viruses infecting cucurbits that can be used for RT-PCR (Gyoutoku et al. 2009, Kwon et al. 2014, Okuda et al. 2007). However, it is unknown whether previously published primer sets will match the sequences of the viral isolates from Okinawa because of the small number of sequences used in primer design. Therefore, it is better to use sequences common to many isolates. In this study, we designed new primer sets for detecting BPYV and KGMMV and performed a field survey of viruses infecting squashes in Okinawa Prefecture, Japan. To the best of our knowledge, this study reports the first detailed field survey of viruses isolated from squashes and cucurbitaceous weeds in Okinawa.

Materials and methods

1. Virus samples

A total of 138 samples of squash plants and nearby weeds were collected from Okinawa (Fig. 1) between December 2019 and January 2020. We collected both squash leaf samples that showed mild or severe mosaic symptoms and asymptomatic leaves from each field. On Okinawa Main Island, we collected 25 and 26 squash samples from fields A and B, respectively, and on Miyako Island, we collected 28, 9, 20, and 14 squash samples from fields C, D, E, and F, respectively. Moreover, we collected seven weed samples that showed necrotic spots and/or chlorosis symptoms. These weed samples included four from *Trichosanthes* sp. and three from *Bryonopsis laciniosa* and were collected from sites adjacent to squash fields C and E on Miyako Island (Fig. 1). Finally, on Ishigaki Island, we collected nine squash samples from field G. All samples were stored temporarily in silica gel in the field and were placed in long-term storage at −80°C until nucleic acid extraction.

2. Primers

To design primers to specifically detect BPYV and KGMMV, we collected all full and partial sequences of the viruses deposited in the DDBJ/ENA/NCBI database

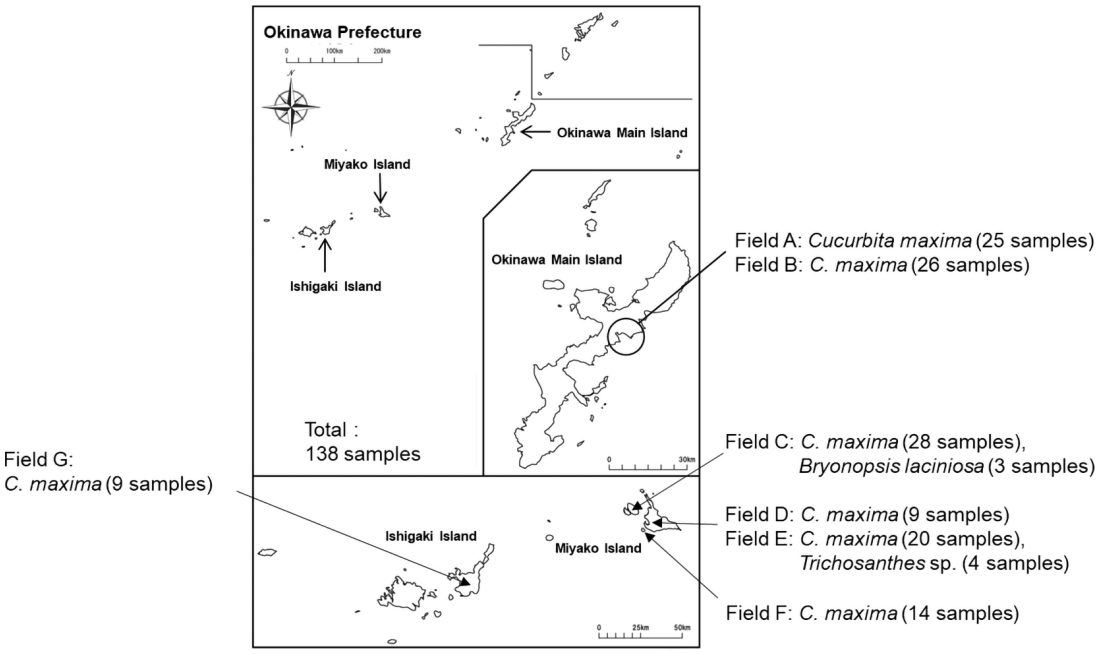

Fig. 1. Locations of sampling sites of squashes and weeds used in this study
Shown are maps of southwest Japan, including Ishigaki Island, Miyako Island, and Okinawa Main Island.

in 2019. These sequences were then aligned using MEGA X software (Kumar et al. 2018), and specific primers were then designed using Primer 3 (Untergasser et al. 2012). Previously reported primers were used to detect CCYV, CGMMV, CMV, MYSV, PRSV, WMV, and ZYMV (Gyoutoku et al. 2009, Okuda et al. 2007). All primers used for detecting viruses are listed in Table 1.

3. Reverse transcription-polymerase chain reaction (RT-PCR)

A small leaf piece was placed in a 2 mL tube and homogenized using 0.5 mm zirconium beads and a multibead shocker (Yasui Kikai, Osaka, Japan). Next, total RNA was extracted from each infected plant using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) or a Trizol reagent (Applied Biosystems, CA, USA), with all procedures performed as per the manufacturer's instructions. Next, one-step RT-PCR was carried out using the Prime Script One-step RT-PCR kit version 2 (TaKaRa Bio, Shiga, Japan) under the following conditions: 30 min at 50°C, 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. RT-PCR was performed using a T100 thermal cycler (BIORAD, CA, USA). All experiments were performed by single-plex RT-PCR using a primer set specific for each virus. After amplification, amplified fragments were separated by electrophoresis using LabChip GXII (Caliper Life Sciences, MA, USA).

4. Sequencing

RT-PCR products were extracted from gels using a QIAquick Gel extraction kit (Qiagen). The nucleotide sequences of the purified RT-PCR products were then determined by the primer walking method using a BigDye Terminator version 1.1 Cycle Sequencing Kit and an Applied Biosystems Genetic Analyzer DNA model 3130 (Applied Biosystems).

5. Statistical analyses

JMP software version 9.0.0 was used for all statistical analyses (SAS Institute Inc., NC, USA). Pearson's chi-square tests were used to investigate the relationship between the presence of symptoms and the virus detected.

Results and discussion

In this study, we designed new primers for detecting BPYV and KGMMV in cucurbit plants, including squashes. RT-PCR was then used to detect each virus. Although a nonspecific amplification product (ca. 480 bp)

Table 1. List of primers used for detecting viruses via RT-PCR

Virus[a]	Primer name	Sequence (5′ to 3′)	Product size	Target gene	Reference
BPYV	BP-3F	GATGCAAGCGAAGGCTGG	1,000	Heat shock protein	This study
	BP-3R	TGCCACGTCTTGTTGGACT			
CCYV	CCYV-HSP-F	TGCGTATGTCAATGGTGTTATG	450	Heat shock protein	Gyoutoku et al. (2009)
	CCYV-HSP-R	ATCCTTCGCAGTGAAAAACC			
CGMMV	mu-CG4F	CGATAAGTTGCTCCCTAAC	1,013	Movement protein	Okuda et al. (2007)
	mu-CG4R	CGTAGGATTGCTAGGATCTAC			
CMV	mu-CM4F	GCGGATGCTAACTTTAGAG	539	Coat protein	Okuda et al. (2007)
	mu-CM4R	TGCTCGATGTCAACATGAA			
KGMMV	KG-1F	CTCTGGACACACAAGACAAACG	619	131 kDa replicase	This study
	KG-1R	GCAGCGAAGAGAACCTTGATGTTT			
MYSV	mu-MY4F	CATTCTGTTGTTTGATGGAAC	888	Nucleocapsid protein	Okuda et al. (2007)
	mu-MY4R	TCCTAAGTAAACACCATGTCTAC			
PRSV	mu-PR4F	GAATGTCCTGAACTTGAATACA	768	Nuclear inclusion b protein	Okuda et al. (2007)
	mu-PR4R	TGGGTGAATGGCAATACA			
WMV	mu-WM4F	AGGGAATCTGGAATGGTT	283	Nuclear inclusion b protein	Okuda et al. (2007)
	mu-WM4R	GGAGTTAAAGAACTGTCGAAC			
ZYMV	mu-ZF4F	GGATAAATTGATGAGAGCATTA	410	Nuclear inclusion b protein	Okuda et al. (2007)
	mu-ZF4R	TGTCAAGTAAGCCGCTATC			

[a] Abbreviations of virus names: beet pseudo-yellows virus (BPYV), cucurbit chlorotic yellows virus (CCYV), cucumber green mottle mosaic virus (CGMMV), cucumber mosaic virus (CMV), kyuri green mottle mosaic virus (KGMMV), melon yellow spot virus (MYSV), papaya ringspot virus (PRSV), watermelon mosaic virus (WMV), and zucchini yellow mosaic virus (ZYMV)

was observed during RT-PCR using the BPYV primers, the size of the product differed from that of the positive sample (Fig. 2 (A)). We did not find nonspecific amplification during RT-PCR using the primers for KGMMV (Fig. 2 (A)). In the past, Okuda et al. (2007) developed a multiplex RT-PCR method using primers designed to detect viruses infecting cucurbit plants. However, only a limited number of nucleotide sequences were used to design these primers. Consequently, the RT-PCR method may not be able to detect all virus mutants, especially BPYV and KGMMV. Moreover, nonspecific amplified products were observed when we attempted RT-PCR (data not shown). Therefore, 12 sequences of BPYV reported from Canada, Greece, Japan, South Africa, and USA were used to identify consensus sequences that we could design primers. We then designed new primers in the heat shock protein genes of these consensus sequences. Similarly, a nonspecific amplified product was observed when attempting to detect BPYV. Despite the existence of this product, the RT-PCR method was still able to distinguish between positive and negative results for BPYV infection. Thus, we conclude that the primer sets designed for BPYV and KGMMV were useful for the detection of both viruses.

Multiplex RT-PCR methods can detect several plant viruses quickly, reliably, and cheaply because these methods detect multiple virus species simultaneously (Pallás et al. 2018, Tanaka et al. 2022). Several studies have reported using multiplex RT-PCR to detect cucurbit-infecting viruses (Okuda et al. 2007, Kwon et al. 2014). However, the primer sets designed in this

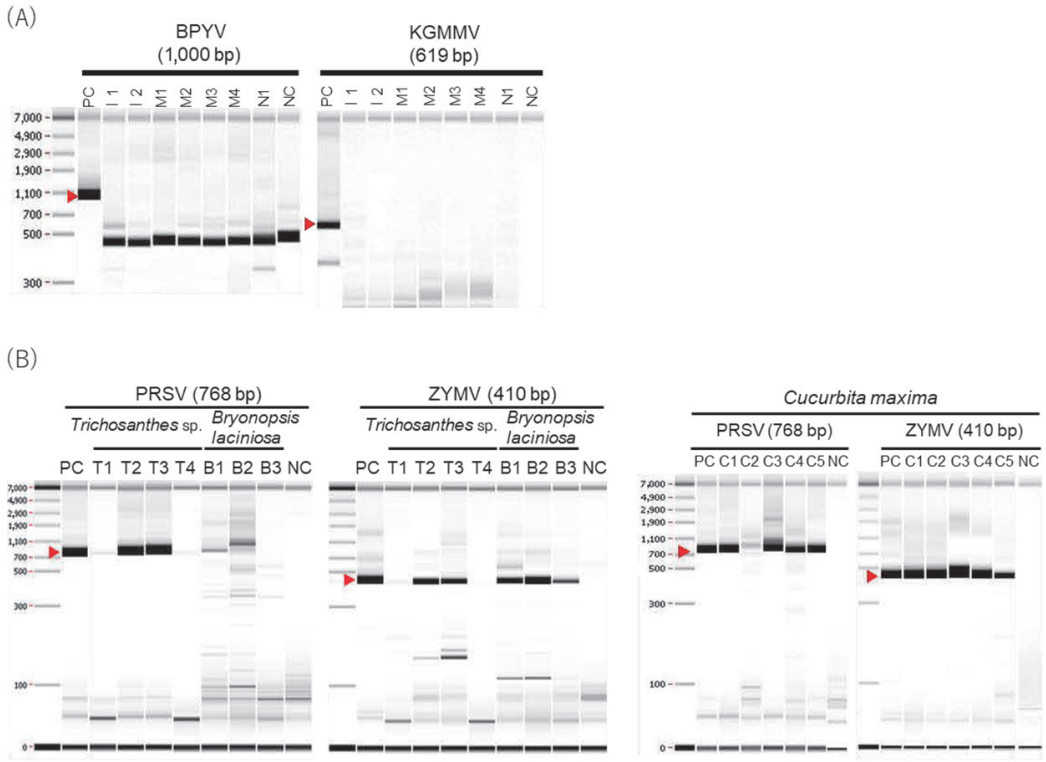

Fig. 2. Electrophoresis image of reverse transcription-polymerase chain reaction (RT-PCR) products using LabChip GXII (Caliper Life Sciences, MA, USA)

The upper image shows detection for beet pseudo-yellows virus and kyuri green mottle mosaic virus using newly designed primer sets (A). Shown are a lane positive control (PC, i.e., total RNA extracted from BPYV and KGMMV obtained from cucumbers grown in Saitama and Tokushima prefectures, respectively); Lanes I1 and I2, Ishigaki samples; Lanes M1-M4, Miyako samples; Lane N1, a sample collected from Okinawa Main Island; Lane NC, negative control (i.e., total RNA extracted from a healthy squash leaf).

The lower image shows the results of the detection for papaya ringspot virus (PRSV) and zucchini yellow mosaic virus (ZYMV) from *Trichosanthes* sp., *Bryonopsis laciniosa*, and *Cucurbita maxima* (B). PC and NC lanes refer to a positive control (i.e., PRSV and ZYMV collected from squashes in Okinawa) and a negative control (water), respectively. Lanes T1-I2, *Trichosanthes* samples; Lanes B1-B3, *Bryonopsis laciniosa* samples; and Lanes C1-C5, *C. maxima* samples growing near the weeds (Field E). Red arrowheads indicate the expected sizes of the RT-PCR products.

study could not be used for multiplex RT-PCR tests of cucurbit-infecting viruses because a nonspecific amplification product was observed during BPYV detection.

When sampling, we collected squash leaves both with and without symptoms. Total RNA was extracted from all 131 squash samples, and RT-PCR was performed using specific primers for the target viruses. Single ZYMV infection was observed in all fields, whereas single infection by PRSV was observed in only three fields (i.e., B, E, and G; Table 2). In addition, mixed infection by ZYMV and PRSV was observed in four fields (i.e., B, D, E, and G; Table 2). Other viruses, including BPYV, CGMMV, CMV, CCYV, WMV, MYSV, and KGMMV, were not detected (data not shown). We also examined the relationship between the appearance of symptoms and the virus detected. In this study, the numbers of plants infected with both ZYMV and PRSV and with only PRSV were pooled because they were fewer than five in number. Statistical analysis revealed a significant difference between viral detection and observed symptoms ($\chi^2 = 10.405$, $P = 0.0055$). These results show that a single ZYMV infection could be significantly detected from the appearance of leaves showing severe mosaic symptoms. This finding suggests that ZYMV is a dominant virus affecting squashes in Okinawa (Table 2). Moreover, these results are consistent with those from study by Ohtsu et al. (1985), in which PRSV was isolated from leaves showing mild and severe symptoms but not from asymptomatic leaves. However, we also note that ZYMV and PRSV were also isolated from asymptomatic plants (Table 2). We speculate that

Table 2. Relationship between the symptoms and detected viruses in *Cucurbita maxima*

Field	Symptom	Detected virus[a]			Not detected[b]	Total
		ZYMV	PRSV	ZYMV&PRSVV		
A	No symptoms	3	0	0	8	11
	Mild mosaic	4	0	0	0	4
	Severe mosaic	10	0	0	0	10
B	No symptoms	0	0	2	1	3
	Mild mosaic	3	1	4	0	8
	Severe mosaic	14	0	1	0	15
C	No symptoms	5	0	0	2	7
	Mild mosaic	8	0	0	0	8
	Severe mosaic	13	0	0	0	13
D	No symptoms	0	0	3	3	6
	Mild mosaic	1	0	2	0	3
	Severe mosaic	0	0	0	0	0
E	No symptoms	3	0	2	5	10
	Mild mosaic	1	1	5	0	7
	Severe mosaic	2	0	1	0	3
F	No symptoms	1	0	0	1	2
	Mild mosaic	6	0	0	0	6
	Severe mosaic	6	0	0	0	6
G	No symptoms	1	0	0	0	1
	Mild mosaic	2	0	1	0	3
	Severe mosaic	2	1	2	0	5
Total	No symptoms	13	0	7	20	40
	Mild mosaic	25	2	12	0	39
	Severe mosaic	47	1	4	0	52

[a] Abbreviations of virus names: papaya ringspot virus (PRSV) and zucchini yellow mosaic virus (ZYMV)

[b] The number of squashes in which none of the nine viruses (i.e., beet pseudo-yellows virus, cucurbit chlorotic yellows virus, cucumber green mottle mosaic virus, cucumber mosaic virus, kyuri green mottle mosaic virus, melon yellow spot virus, PRSV, watermelon mosaic virus, and ZYMV) was detected.

the detection of the virus in asymptomatic plants is related to the timing of the infection. In addition, we detected no other viruses in leaves showing mild and severe mosaic symptoms. Two previous studies found that WMV and CMV were detected in squashes in Japan (Komuro 1956, Yonaha et al. 1977). However, we found no evidence of these viruses infecting squash in this study. Instead, our results indicate that PRSV and ZYMV are the causal agents of mosaic diseases of squashes in Okinawa. PRSV and ZYMV belong to the family *Potyviridae* and the genus *Potyvirus* and are transmitted by aphids in a nonpersistent manner (Gibbs & Ohshima 2010). In Okinawa, squashes are commonly planted in early December and grown until May of the following year. Furthermore, most squashes are cultivated in open fields. Because aphids are more prevalent in Okinawa during this time period than whiteflies or thrips, we speculate that ZYMV and PRSV could be more frequently detected for this reason. Therefore, controlling aphids may be essential for controlling diseases caused by infection with PRSV and ZYMV.

Next, we examined viral infection of nearby weeds, which are an essential reservoir of plant viruses. PRSV and ZYMV were detected by RT-PCR from *Trichosanthes* sp. showing necrotic symptoms (Fig. 2 (B), Lanes T2, T3) but not from *Trichosanthes* sp. showing chlorosis symptoms (Fig. 2 (B), Lanes T1, T4). By contrast, only ZYMV was detected via RT-PCR from *Bryonopsis laciniosa* samples (Fig. 2 (B), Lanes B1-B3). Moreover, the virus species detected in the weeds were identical to those in *C. maxima* samples growing near the weeds (Figs. 1, 2; Table 2). For instance, PRSV and ZYMV were detected in *C. maxima* growing near the affected weeds in field E (Fig. 2B, Lanes C1-C5). Sequence analysis showed that the nucleotide sequences of the RT-PCR products obtained from the weeds were derived from the sequences of PRSV and ZYMV (data not shown). We then deposited these sequences in DDBJ/ENA/GenBank (Accession Nos. LC770990-LC770996). The typical symptoms of leaves of *C. maxima* infected with PRSV, ZYMV, or both PRSV and ZYMV are shown in Figure 3A, B, and C. In addition, an example of necrotic spots and/or chlorosis symptoms on leaves of *Trichosanthes* sp. near squash fields were shown in Figure 3D.

Trichosanthes sp. and *Bryonopsis laciniosa* are common perennial weeds in Japan. Thus, once infected with the virus, the weeds can retain it and are therefore presumed to be the source of transmission. In a previous study, Yonaha et al. (1988) isolated both the trichosanthes mottle virus and WMV from *Trichosanthes rostrata* growing in Okinawa. Moreover, the authors indicated

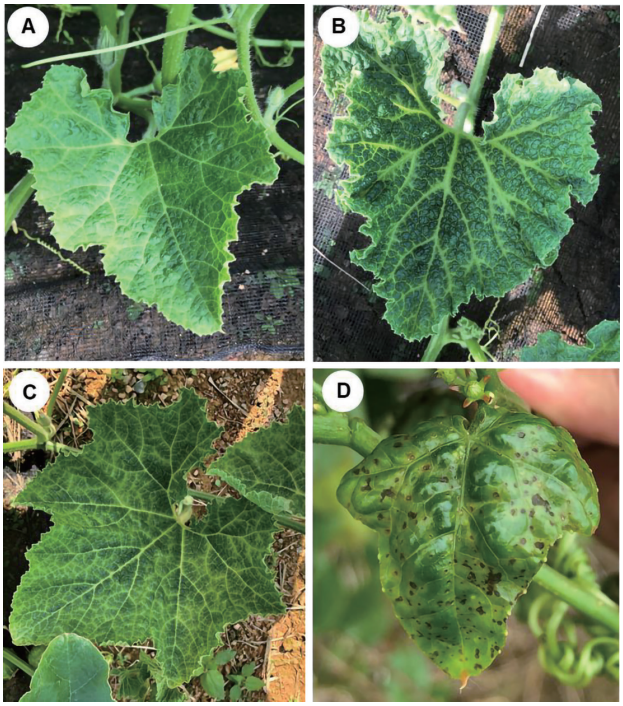

Fig. 3. Typical symptoms observed in *Cucurbita maxima* (A, B, and C) and *Trichosanthes* sp. (D)
The detected viruses included the following: A: papaya ringspot virus (PRSV), B: zucchini yellow mosaic virus (ZYMV), and C and D: both PRSV and ZYMV.

that these viruses were transmitted by two identifiable aphid species, namely, *Myzus persicae* and *Aphis gossypii*. These results suggest that controlling cucurbitaceous weeds could effectively restrict the spread of viral diseases in squashes. However, whether PRSV and ZYMV are transmitted from weeds to squashes via aphids is unclear. To clarify this hypothesis, we must determine and compare the DNA sequences of PRSV and ZYMV isolated from weedy plants and squashes. In this study, only two viruses, namely, ZYMV and PRSV, were detected from squashes in Okinawa Prefecture. However, because Okinawa is geographically close to Taiwan, there are concerns that the virus may migrate from Taiwan to Okinawa. In Taiwan, cucurbit aphid-borne yellow virus, melon vein-banding mosaic virus, and squash leaf curl Philippines virus have been reported as infecting plants from the family Cucurbitaceae (Huang & Chang 1993, Knierim et al. 2010, Tsai et al. 2007). It is therefore important to investigate whether these viruses are infecting squashes in Okinawa Prefecture.

In conclusion, to the best of our knowledge, this is the first detailed survey of the viruses infecting *C. maxima* in Okinawa. Moreover, the RT-PCR method used in this study can help future surveys of these viruses in all cucurbit cultivation areas, including Southeast Asia.

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