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Comparing Symptoms and Virus Accumulation of Three Tobamoviruses in Cucurbitaceae Hosts

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Abstract

Tobamoviruses that infect cucurbits are significant pathogens in crops of the Cucurbitaceae family, spreading through seed-borne, mechanical, and soil-transmission methods. The cucumber green mottle mosaic virus (CGMMV) and the kyuri green mottle mosaic virus (KGMMV) have been prevalent in Japan since the 1960s. In contrast, the zucchini green mottle mosaic virus (ZGMMV) has only been reported in South Korea and China. To understand the potential impact of ZGMMV on cucurbit production in Japan, we compared the systemic symptoms and virus quantities in infected plants across four cucurbit crops: cucumber, melon, watermelon, and zucchini. Each of these crops was inoculated with each virus. All the viruses caused systemic infections. However, KGMMV generally induced the most severe leaf symptoms and showed the highest accumulation of the coat protein followed by ZGMMV and CGMMV in terms of symptom severity and protein accumulation. These findings, although derived from a small-scale, short-term experiment, could be instrumental in assessing the risk these tobamoviruses pose to cucurbit production.

Discipline: Agricultural Environment
Additional key words: coat protein, cucurbit, RT-PCR, symptom, tobamovirus

Introduction

The Cucurbitaceae family, also known as cucurbits, encompasses approximately 965 species across 95 genera. This family includes several economically significant crops such as cucumbers and gherkins (*Cucumis sativus*), cantaloupes and other melons (*Cucumis melo*), watermelons (*Citrullus lanatus*, *C. colocynthis*), and pumpkins, squash, and gourds (*Cucurbita maxima*, *C. pepo*, *C. moschata*, etc.). These crops are cultivated globally, with 2023 production levels reaching 97.8, 29.5, 104.9, and 23.7 million tons, respectively (FAOSTAT 2024).

Cucurbit production can be threatened by diseases caused by various cucurbit-infecting viruses. Tobamoviruses (Family *Virgaviridae*, Genus *Tobamovirus*) are particularly damaging due to their strong mechanical and seed-transmissibility. They cause severe damage, including leaf mosaics, fruit distortion, and growth inhibition. Out of the 37 tobamoviruses recognized by the International Committee on Taxonomy of Viruses (ICTV MSL36), five infect cucurbits: cucumber fruit mottle mosaic virus (CFMMV) (*Tobamovirus maculafructi*), cucumber mottle virus (CuMoV) (*Tobamovirus cucumeris*), cucumber green mottle mosaic virus (CGMMV) (*Tobamovirus viridimaculae*), kyuri green mottle mosaic virus (KGMMV) (*Tobamovirus kyuri*), and zucchini green mottle mosaic virus (ZGMMV) (*Tobamovirus cucurbitae*).

CFMMV and CuMoV have only been reported in Israel and Miyazaki Prefecture, Japan, respectively, and have only been observed temporarily (Antignus et al. 2001, 2005; Orita et al. 2007). In contrast, CGMMV and KGMMV have a broader distribution in Japan and other countries. CGMMV, initially described as “Cucumber virus 3” and “Cucumber virus 4” by Ainsworth (1935) (Francki et al. 1986), became endemic in several Japanese prefectures in 1968, causing mosaic and fruit deterioration on watermelon (Komuro et al. 1971). It was later found in

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melons in Shizuoka Prefecture in 1970 (Ohsawa 1990). Since then, CGMMV has been recognized in various cucurbit crops worldwide, particularly in Europe and Asia (Webster & Jones 2018). It has also raised significant concerns in the melon industry in Australia and California since its first occurrence a decade ago (Australian Government 2017, Pitman et al. 2022). Seed transmission of CGMMV has been reported at rates of 2.25%, 2.83%, and 2% on watermelon, melon, and bottle gourd, respectively (Choi et al. 2001, Wu et al. 2011). KGMMV was first reported on cucumber fields in wide areas of Western Japan in 1966 (Inoue et al. 1967) and was initially thought to be a strain of CGMMV. However, subsequent studies revealed that KGMMV is a distinct tobamovirus from CGMMV (Francki et al. 1986, Tochihara & Komuro 1974). KGMMV has been found in Indonesia, Korea, and Turkey on melon and other cucurbits, as well as cucumber (Balsak 2023, Daryono et al. 2005, Kim et al. 2009). The seed-to-seedling transmission rate of KGMMV in cucumber and melon plants is 0.5% (Balsak 2023). ZGMMV was first isolated from zucchini squash (*C. pepo*) showing leaf mosaics and fruit abnormalities in South Korea (Ryu et al. 2000) and was also found on greenhouse-grown bottle gourd (*Lagenaria siceraria*) plants in China (Li et al. 2018). There are indications that it may have also occurred in Saudi Arabia (Al-Dosary et al. 2012). Inoculation experiments have demonstrated that ZGMMV has a broad host range in cucurbits, including cucumber, melon, and watermelon (Yoon et al. 2002).

While the ZGMMV has not yet been reported in Japan, it is important to consider the potential risk of its introduction and spread within the country. This is particularly relevant given that cucurbits are widely cultivated in Japan, and a significant portion of the seeds used for cultivation are imported from abroad, which could potentially introduce ZGMMV. In light of these factors, the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan has classified ZGMMV as a hazardous plant pathogen that requires appropriate risk management measures (Yokohama Plant Protection Office 2020). However, the extent of the risks posed by ZGMMV, particularly in comparison to the widely distributed CGMMV and KGMMV, remains unclear due to the lack of comparative studies on the damage caused by these three viruses. Such information would be invaluable in developing a control strategy for ZGMMV and in determining the priority among cucurbit-infecting tobamoviruses. In this study, we created an infectious ZGMMV based solely on its nucleotide sequence and conducted inoculation experiments on four major cucurbit crops: cucumber, melon, watermelon, and zucchini. By comparing symptom severity and the amount of virus accumulation among the viruses and crops, we discuss the risk of ZGMMV compared to CGMMV and KGMMV.

Materials and methods

1. Construction of ZGMMV infectious cDNA

Given that ZGMMV has not been observed in Japan, strict precautions were taken in advance by notifying the Yokohama Plant Protection Office, MAFF about the production and utilization of the ZGMMV infectious cDNA clone. All experiments were conducted with careful attention to avoid any potential hazards. The infectious cDNA of ZGMMV was created as detailed in Figure 1. The nucleotide sequence of the clone was derived from a South Korean ZGMMV isolate (ZGMMV-K, GenBank accession No. NC_003878), which spanned 6,513 nt (Ryu et al. 2000, Yoon et al. 2002). Partial cDNA fragments, namely ZG_1 (nt 1-2278, with an additional T7 RNA polymerase promoter sequence at the 5' terminus), ZG_2 (nt 2264-4495), and ZG_3 (nt 4481–6513, with an additional SphI restriction site at the 3' terminus), were synthetically constructed with 15-nt overlaps between the fragments (Integrated DNA Technologies, Singapore). Each fragment was PCR-amplified using the primers listed in Table 1 and purified via gel electrophoresis. Despite attempts to insert the three full-length fragments into a pUC19 vector using the In-Fusion HD Cloning Kit (TakaraBio, Otsu, Shiga, Japan) and cloning them into Escherichia coli JM109, no clone with infectivity was obtained, consistent with previous findings (Yoon et al. 2002). Consequently, the three fragments were assembled to full-length using overlap extension PCR, as illustrated in Figure 1. Fragments ZG_4 (nt 1–3020) and ZG_5 (nt 3000–6513) were PCR-amplified using a mixture of PCR-amplified fragments ZG_1, ZG_2, and ZG_3 as template. Subsequently, fragment ZG_6 was amplified using a mixture of ZG_4 and ZG_5 as templates along with primers ZGET7-corrected and ZG3SPH-corrected. Finally, ZG-6 was re-amplified via nested PCR using primers ZGET7-corrected-nest and ZG3SPH-corrected-nest. PCR amplification utilized the KOD One PCR Master Mix (Toyobo, Osaka, Japan) under the following thermal cycling conditions: 35 cycles of 98°C for 3 s, 55°C for 5 s, and 68°C for 1 min. The resulting amplicons, approximately 6.5 kb in length, were purified via gel electrophoresis, digested with SphI (SphI-HF, New England Biolabs, Ispwich, UK), and employed for in vitro transcription.

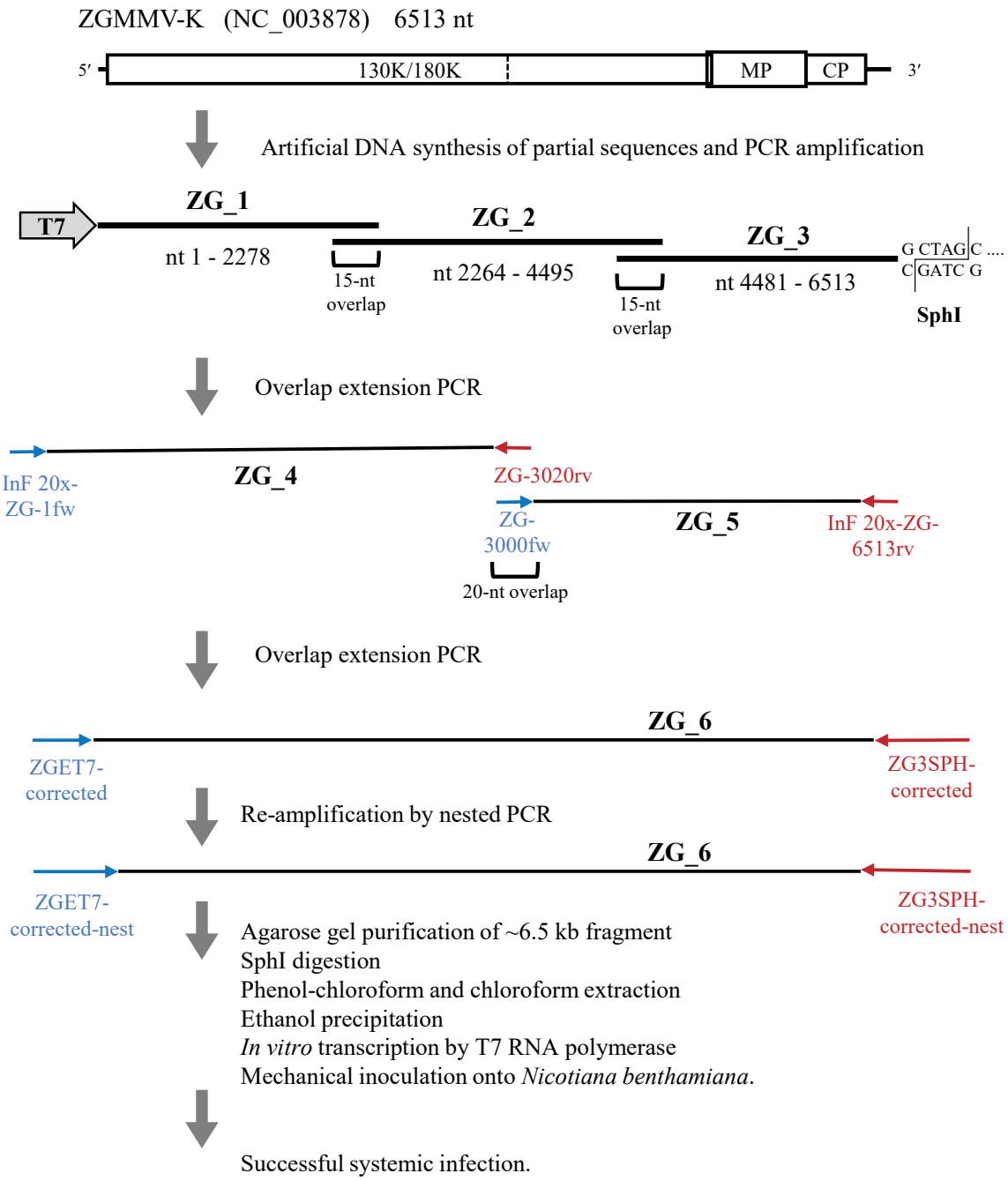

Fig. 1. Flow of construction of the infectious transcripts of the zucchini green mottle mosaic virus (ZGMMV)
The forward and reverse primers are represented by blue and red arrows, respectively.

2. In vitro transcription, inoculation, and virion purification

In vitro transcripts were generated from the full-length cDNA previously described using the RiboMax Large Scale RNA Production System (T7) (Promega, Tokyo, Japan). This reaction was carried out in the presence of a 3′-O-Me-m^{7}G(5′)ppp(5′)G RNA Cap Structure Analog (New England Biolabs). The transcribed RNA was then mechanically inoculated onto the expanded leaves of *Nicotiana benthamiana* plants using a glass rod and carborundum powder. The inoculated plants were cultivated in a growth chamber (LH-241PFDT-S, Nippon Medical & Chemical Instruments, Osaka, Japan) at a temperature of 24°C, with a photoperiod of 16 h of light and 8 h of darkness, for 14 days. Non-inoculated upper leaves that exhibited

Table 1. Primers used in this study

Primer name	Sequence (5′ to 3′)	Purpose
InF-ZGMMV-1fw	GGTACCCGGGGATCCTAATACG	Amplification of fragment ZG_1
ZGMMV-2278rv	AGATTGCAGGTCTCCATCATATGG	
ZGMMV-2264fw	GGAGACCTGCAATCTCAGACTC	Amplification of fragment ZG_2
ZGMMV-4527rv	TTATGGCCTATCTCCCAGACCC	
ZGMMV-4513fw	GGAGATAGGCCATAAGCGTACC	Amplification of fragment ZG_3
InF-ZGMMV-6513rv	CAGGTCGACTCTAGAGCATGCTGGGG	
InF-20x-ZGMMV-1fw	AGCTCGGTACCCGGGGATCCTAATACG	Amplification of fragment ZG_4
ZGMMV-3020rv	GAATTTGTTTGGTATCCCCAAAAATCTT	
ZGMMV-3274fw	AAGCAGACCCTTCTCAAGGCTGGT	Amplification of fragment ZG_5
InF-20x-ZGMMV-6513rv	GCCTGCAGGTCGACTCTAGAGCATGCTGGGG	
ZGET7-corrected	GAGAGAATTCTAATACGACTCACTATAGAAAGAGACAAACATTAAAC	Amplification of fragment ZG_6
ZG3SPH-corrected	GAGAGCATGCTGGGGTCCTTACCCAGGACAAAG	
ZGET7-corrected-nest	GAGAGAATTCTAATACGACTCACTATAGAAAGAGACAAACATTAAAC ATAC	Re-amplification of ZG_6 by nested PCR
ZG3SPH-corrected-nest	GAGAGCATGCTGGGGTCCTTACCCAGGACAAAGGGGG	
ZGMMV-1fw	GAAAGAGACAAACATTAAAC	Sequencing
ZGMMV-300rv	TGTACAGATAAAGCGGATCC	
ZGMMV-600fw	GAAAGAGACAAACATTAAAC	
ZGMMV-900rv	TCATCTTCGAACTGAAAATT	
ZGMMV-1200fw	TGCAAGGATTTTTCTCTGGT	
ZGMMV-1500rv	CCCTGAGGTTGTGTCACGAT	
ZGMMV-1800fw	ACTTCTGTCATCATCATGAC	
ZGMMV-2100rv	TCGGTAACAGCCTTTCTGTT	
ZGMMV-2400fw	TCGTCAGATCTTCGTGGTCT	
ZGMMV-2700rv	GTAAATTTTGCAGTGCAGTC	
ZGMMV-3000fw	TGGGGATACCAAACAAATTC	
ZGMMV-3300rv	GCCTTGAGAAGGGTCTGCTT	
ZGMMV-3600fw	TATTGCCCGTATCTAAAGCG	
ZGMMV-3900rv	GGAGTATTAAAATTACGTTT	
ZGMMV-4200fw	TTTATCATTCCAAAGTAGTT	
ZGMMV-4500rv	GTACGCTTATGGCCTATCTC	
ZGMMV-4800fw	AGGGCTGTATTGTATATCCT	
ZGMMV-5100rv	CTTCACACCTAACTTGCTAA	
ZGMMV-5400fw	CAAGTTCTCAGTCGCCGCAT	
ZGMMV-5700rv	AGGAACTGAATCGAAGAAGG	
ZGMMV-6000fw	GTGAGTCGCTCGTTGGTTTG	
ZGMMV-6300rv	TCAAAAGACGCTCTATCAAA	
ZGMMV-6513rv	TGGGGTCCTTACCCAGGACA	

mottle and leaf curling were harvested, and the ZGMMV virion was purified via polyethylene glycol precipitation, following the method described by Chapman (1998). The purified virion preparation was then analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie brilliant blue (CBB). The preparation was stored at −80°C for future use. The complete nucleotide sequence, excluding approximately 20 nucleotides at both the 5′- and 3′-termini, was determined through direct sequencing of RT-PCR products derived from the genomic RNA, which was obtained from purified virion preparation with SDS, phenol, and chloroform-based extraction as described by Chapman (1998), using the primers listed in Table 1.

3. Inoculation of plants and symptom observation

The CGMMV isolate W-1 (MAFF104026, referred to as CGMMV-W) and the KGMMV isolate Cu 66-1 (MAFF104025, referred to as KGMMV-C) were sourced from the Gene Bank Project, NARO. These isolates were propagated on cucumber plants, and the virion was purified using the method previously described. The Cucurbitaceae plants used in this study included the following varieties: cucumber cv. Courage (Tokiwa Co., Ltd., Yoshimi, Saitama, Japan), melon cv. Miyabi-Syunjyuukei (Yokohamaueki Co., Ltd., Yokohama, Kanagawa, Japan), watermelon cv. Ibuki (Takii Co., Ltd., Kyoto, Japan), and zucchini cv. Green-tosca (Sakata Seed, Yokohama, Japan). Each plant was grown individually in a plastic pot with a diameter of 9 cm, filled with nursery soil. The plants were inoculated with the purified virion solution (100 ng/µl) onto two cotyledons and were grown for up to four weeks in a growth chamber, as previously described.

4. DAS-ELISA of ZGMMV

From the uppermost expanded leaf of each plant (in total of two inoculated and one non-inoculated plant for each crop), two leaf disks, each 6 mm in diameter, were harvested from the uppermost expanded leaf of each inoculated plant and stored at –20°C. These samples were then subjected to DAS-ELISA using a Zucchini Green Mottle Mosaic Virus Set from Loewe, Sauerlach, Germany, in accordance with the manufacturer's instructions. Following this, the samples were incubated with a p-nitrophenyl phosphate substrate (pNPP tablet, Sigma-Aldrich, Darmstadt, Germany) for 1 h at room temperature. The optical density (OD) at 405 nm was then measured.

5. CP detection by SDS-PAGE

Two leaf disks, obtained as described above, were placed into a 2-ml centrifuge tube along with two zirconia beads (5 mm in diameter) and 100 µl of 1× tris-buffered saline supplemented with a proteinase inhibitor cocktail, CompleteMini (Roche Diagnostics, GmbH, Mannheim, Germany). The mixture was macerated by vibrating at 50 Hz for 1 min using a TissueLyzer LT (Qiagen). Following centrifugation at 20,000 ×g for 5 min, the supernatant was transferred into a new centrifuge tube and combined with an equal volume of 2X SDS-PAGE sample buffer (Nacalai, Kyoto, Japan). The mixture was then heat-denatured by incubating at 98°C for 3 min. The total soluble proteins were separated via SDS-PAGE using a 5%-20% gradient polyacrylamide gel (ATTO, Tokyo, Japan) and visualized by staining with CBB Stain One Super (Nacalai).

6. RT-PCR detection of viruses

Total RNA was isolated from two leaf disks using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), as described by Matsushita et al. (2024). The RT-PCR process was carried out with the following primers: for CGMMV (designed from the sequence of GenBank accession No. AB369274): CG-W-600fw (5′-TGTTCAGACGTTTTCCAAGA-3′) and CG-W-900rv (5′-GAAAGTAAGTACGCATCACGAT-3′), For KGMMV (designed from AJ295948): KGC1-600fw (5′-GACGCATTCCGTAGGTACCA-3′) and KGC1-900rv (5′-AACTATGCGTGTAATGTAAAGTAGA-3′), for ZGMMV:ZG-F(5′-CCGAGCAGATGCGTGTGGTGAC-3′) and ZG-R (5′-CCGATCTGCTCGCACGGAATG-3′). These primers were used following the methodology outlined by Li et al. (2018). The One-Step RT-PCR Kit (Qiagen) was utilized for the procedure. Thermal cycling was set with an initial step at 50°C for 30 min, followed by a denaturation step at 95°C for 15 min. This was followed by 35 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 50 s (extension). A final extension was performed at 72°C for 10 min. The PCR products were then separated by electrophoresis on 2% (w/v) agarose gels.

Results

1. Construction of the infectious ZGMMV cDNA

In line with the findings of Yoon et al. (2002), our efforts to clone the full-length ZGMMV-K cDNA into *E. coli* were unsuccessful. However, the *in vitro* transcripts that were prepared from the PCR-amplified fragments proved to be infectious. Non-inoculated upper leaves of two out of the two inoculated *N. benthamiana* plants tested positive for ZGMMV by RT-PCR analysis with primers ZGMNV-1200fw and ZGMMV-1500rv (Table 1) (data not shown) and induced mosaic symptoms (Fig. 2a). We purified ZGMMV virions from the symptomatic leaves and analyzed them using SDS-PAGE. This analysis revealed the presence of a CP with an estimated molecular mass of 17.1 kDa (Fig. 2b). We determined the nucleotide sequences of the virion RNA from nt 21 to 6493. They were found to be almost identical to those of NC_003878, with two exceptions. One was a synonymous nucleotide substitution from U to C at nt 2639 in the 131/189K replication protein gene. The other was a nonsynonymous substitution at nt 5627G to A, which resulted in an amino acid substitution of 185Val to Ile in the movement protein. We hypothesized that these mutations would have minimal or no impact on the viral performance, such as its accumulation and virulence in the infected plants. Therefore, the virion was used as an inoculum for

further analysis.

2. DAS-ELISA

In an initial experiment, we inoculated *N. benthamiana*, cucumber, watermelon, and zucchini plants with a purified virion preparation of ZGMMV-K. We then evaluated the accumulation of ZGMMV in the upper leaves at 14 days post-inoculation (dpi) using DAS-ELISA (Fig. 3). We observed ODs in two plants, each of *N. benthamiana* and watermelon. In contrast, zucchini and cucumber plants yielded moderate and significantly lower ODs, respectively. At 14 dpi, we noted mosaic symptoms on *N. benthamiana* and watermelon plants, very mild chlorosis on zucchini plants, and no symptoms on cucumber plants. By 28 dpi, zucchini and cucumber plants began to exhibit chlorotic spots and mild

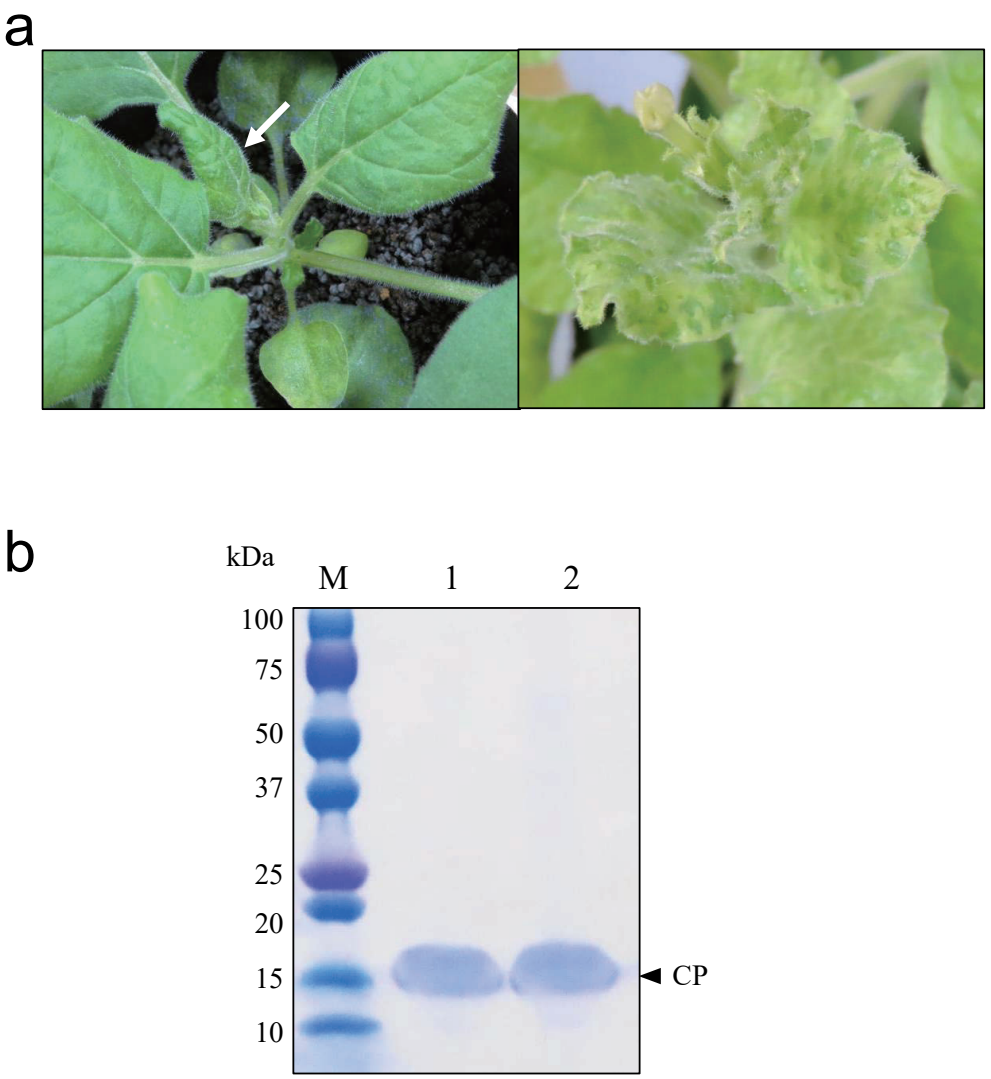

Fig. 2. Infectivity of artificially constructed transcripts of zucchini green mottle mosaic virus (ZGMMV)

(**a**) Systemic infection of ZGMMV on *Nicotiana benthamiana* plants that were inoculated with *in vitro* transcripts of full-length ZGMMV, showing outward leaf curling (arrow) at 11 days post inoculation (dpi) (left) and mosaic at 32 dpi (right). (**b**) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of ZGMMV virion purified from upper leaves of the transcripts-inoculated *N. benthamiana*. Lane M, molecular size marker (Precision Plus Dual Color Standards, BioRad); lanes 1 and 2, virion preparation purified by polyethylene glycol precipitation from non-inoculated upper leaves of the *N. benthamiana* inoculated with the transcripts. Arrow indicates the ZGMMV coat protein (CP), with an expected molecular mass of 17.1 kDa.

chlorosis, respectively (data not shown). Based on these observations, we inferred that while cucumber can systemically host ZGMMV, it is not an ideal host for this virus.

3. Symptom comparison

We mechanically inoculated cucumber, melon, watermelon, and zucchini plants with virion preparations of CGMMV-W, KGMMV-C, and ZGMMV-K. We then compared the symptoms on the upper leaves of these plants up to 20 days post-inoculation (dpi) (Fig. 4). On cucumber plants, KGMMV induced the most severe symptoms, which included yellow spots, mosaic patterns, and leaf malformation. The symptoms induced by CGMMV and ZGMMV were less severe (Fig. 4a). On melon plants, all three viruses induced severe systemic symptoms (Fig. 4b). On watermelon plants, ZGMMV-inoculated plants exhibited much milder symptoms compared to those inoculated with CGMMV and KGMMV (Fig. 4c). On zucchini plants, KGMMV caused more yellow spots than ZGMMV. Interestingly, no symptoms were observed on zucchini plants inoculated with CGMMV (Fig. 4d). In general, KGMMV induced the most severe symptoms across the four cucurbit crops.

4. CP accumulation

The accumulation of each virus in the symptomatic upper leaves was estimated by analyzing the CPs present in the total soluble proteins of the infected upper leaves. This analysis was conducted using SDS-PAGE and CBB staining (Fig. 5a). Among the viruses, KGMMV CP was found to be the most abundant in cucumber, melon, watermelon, and zucchini plants when compared to CGMMV and ZGMMV CPs. CGMMV CP was abundantly detected in two melon plants and one out of two watermelon plants. However, it was below the detectable level in cucumber and zucchini plants. Similarly, ZGMMV CP was detected in two melon plants, and one out of two watermelon and zucchini plants, but its levels were lower than those of KGMMV. Therefore, it was observed that the accumulation levels of CP primarily correlated with the severity of the systemic symptoms in combinations of each virus and host species (Fig. 4). Interestingly, some plants such as zucchini plants inoculated with CGMMV, did not exhibit obvious symptoms nor did they contain detectable levels of CP (Figs. 4, 5a). Despite this, RT-PCR analysis confirmed that all inoculated plants were infected with the inoculated virus (Fig. 5b).

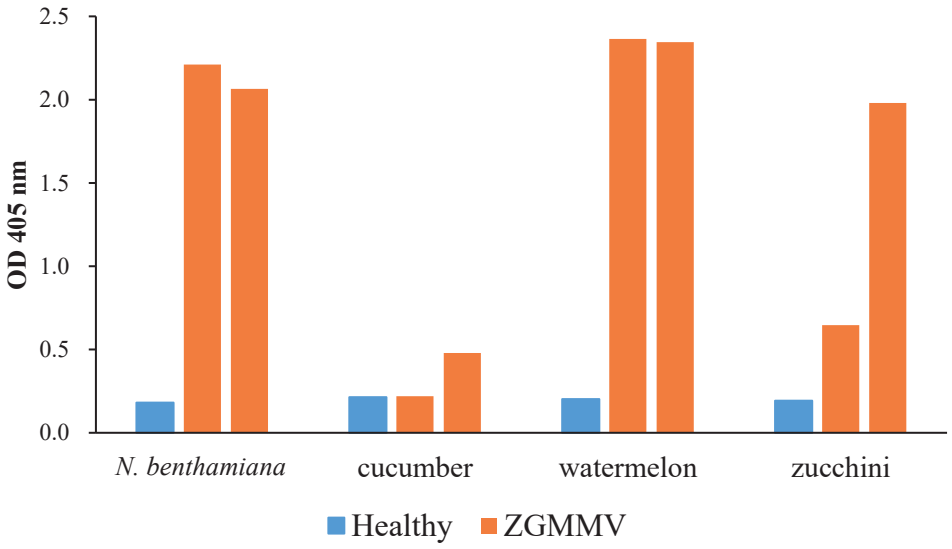

Fig. 3. Evaluation of the accumulation of zucchini green mottle mosaic virus (ZGMMV) by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)
DAS-ELISA was conducted on healthy and zucchini green mottle mosaic virus (ZGMMV)-inoculated plants. The plant species tested include *Nicotiana benthamiana*, cucumber, watermelon, and zucchini. The assay was performed on non-inoculated upper leaves 14 days post-inoculation. The Figure displays the results from one healthy plant and two ZGMMV-inoculated plants.

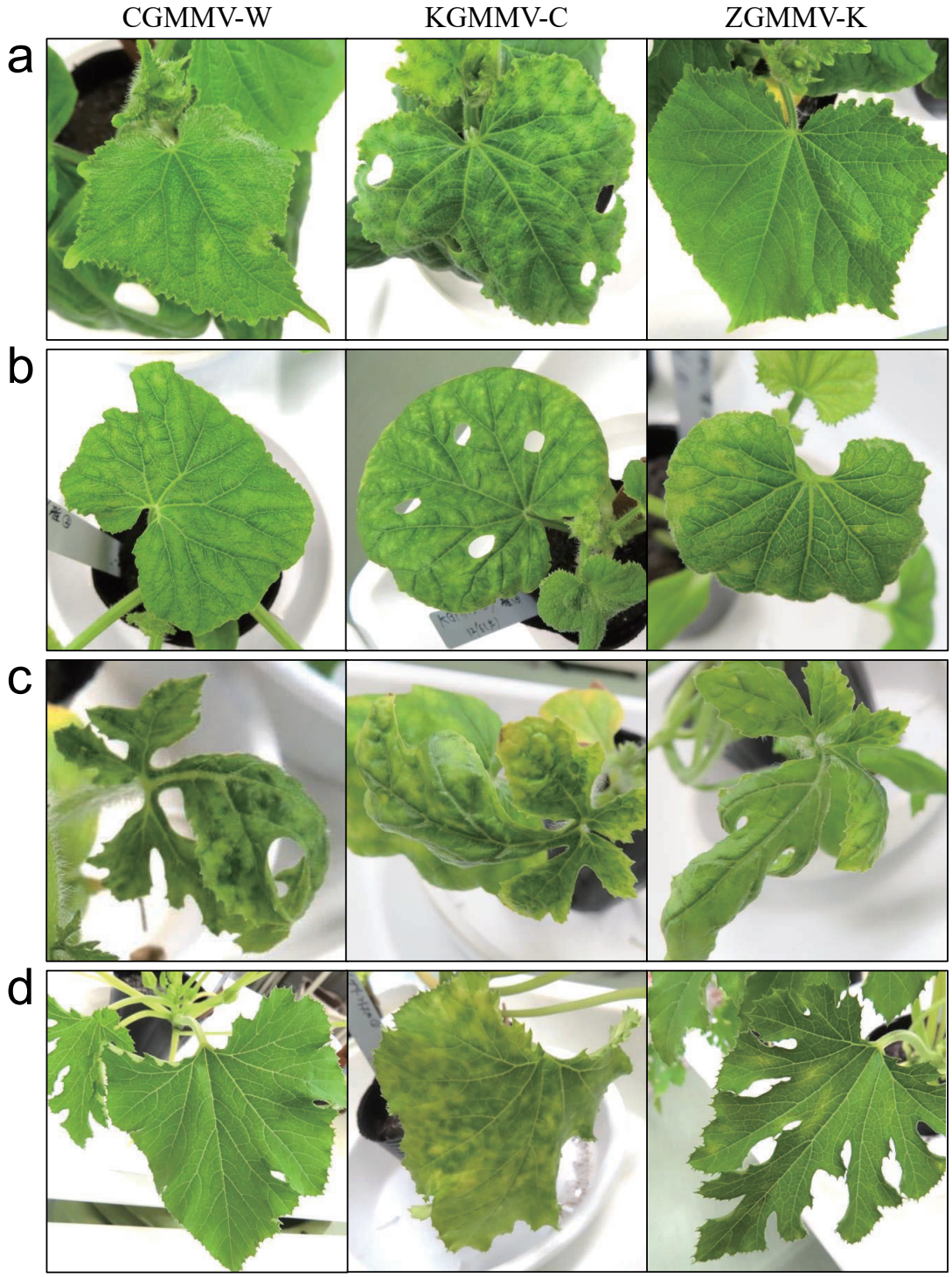

Fig. 4. Comparison of systemic symptoms on cucurbit crops

Systemic symptoms on cucumber (**a**), melon (**b**), watermelon (**c**), and zucchini (**d**) plants inoculated with cucumber green mottle mosaic virus (CGMMV), kyuri green mottle mosaic virus (KGMMV), or zucchini green mottle mosaic virus (ZGMMV), as indicated at the top. Each virus was mechanically inoculated onto cotyledons, and photographs were taken at 15 days post-inoculation (dpi) for **a** and **b**, and at 20 dpi for **c** and **d**. In general, KGMMV induced more severe symptoms than CGMMV and ZGMMV, especially in cucumber and zucchini. Please note that the difference in leaf shape between zucchini plants inoculated with KGMMV (middle) and ZGMMV (right) in Figure 4d did not result from the viral infection but was due to different leaf positions.

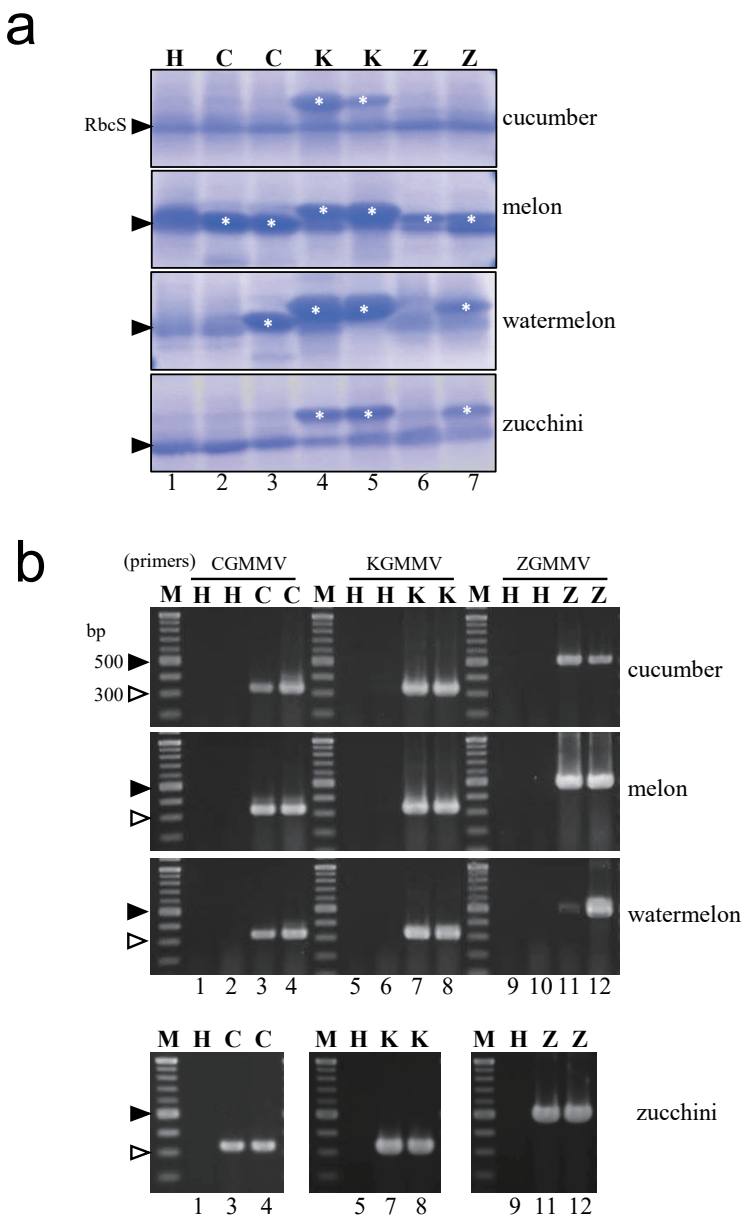

Fig. 5. Comparison of coat protein accumulation on cucurbit crops

(a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of total soluble proteins extracted from the upper leaf of a non-inoculated healthy plant (Lane 1, **H**), or plants inoculated with cucumber green mottle mosaic virus (CGMMV) (2 and 3, **C**), kyuri green mottle mosaic virus (KGMMV) (4 and 5, **K**), or zucchini green mottle mosaic virus (ZGMMV) (6 and 7, **Z**). The inoculated host plants are indicated at the right. Samples were collected 15 days post-inoculation (dpi). The bands of the coat protein of the tobamoviruses (~18 kDa) and of the RuBisCo small subunit (RbcS, ~15 kDa) are indicated by asterisks and arrowheads, respectively. (**b**) RT-PCR detection with primers specific to CGMMV, KGMMV, or ZGMMV, as indicated at the top. At 20 dpi, total RNAs were extracted from the non-inoculated upper leaf of healthy plants (lanes 1, 2, 5, 6, 9, and 10), plants inoculated with CGMMV (3 and 4), KGMMV (7 and 8), or ZGMMV (11 and 12), and were subjected to RT-PCR. Amplicons were separated by 2% (w/v) agarose gel electrophoresis. Lane M: size marker, 1-kb Plus DNA Ladder (New England Biolabs); 500- and 300-bp bands are indicated by solid and open arrowheads, respectively.

Discussion

Understanding the biological properties and the degree of disease severity of a plant pathogen is crucial for developing control strategies and determining its priority. This is especially true for emerging pathogens that are only found in foreign countries. In this study, we attempted to evaluate the risk of ZGMMV by comparing it with CGMMV and KGMMV in terms of symptom severity and CP accumulation in four cucurbit crops. Despite the small- scale of the test, we were able to draw some conclusions. We successfully constructed an infectious clone of ZGMMV through artificial gene synthesis, based on the nucleotide sequence retrieved from a database. The ZGMMV genome was synthesized in three overlapping parts. Given that the cost of gene synthesis increases exponentially as the synthesized sequence becomes longer, synthesizing three 2.2 kb fragments can save approximately 45% of the synthesis cost compared to synthesizing the full genome as a single fragment. Furthermore, overlap extension PCR was successfully applied to assemble the full genome and prepare the template for infectious *in vitro* transcripts without the need for cloning in *E. coli*. This method is a cost-effective way to prepare a virus inoculum when only sequence information is available and bacterial cloning is difficult or impossible.

The DAS-ELISA and SDS-PAGE analysis results of plants inoculated with the ZGMMV virion revealed that ZGMMV accumulates more in melon and watermelon plants and less in cucumber plants compared to zucchini (Figs. 3, 5a). Consistent with this, cucumbers exhibited fewer symptoms, while melon, watermelon, and zucchini plants displayed similar or higher degrees of systemic symptom severity (Fig. 4). These results suggest that ZGMMV could potentially cause agricultural damage to cucurbits, particularly in melon and watermelon. However, when compared with CGMMV and KGMMV, ZGMMV induced milder symptoms and less CP accumulation across the four crops (Figs. 4, 5). This suggests that KGMMV may pose a greater threat than ZGMMV and CGMMV if it invades countries where it is not currently present. It is essential to note that our results were obtained from a small-scale, short-term experiment utilizing a limited combination of virus isolates and cultivars.

The risk level of plant pathogens is influenced by numerous factors, including not only leaf symptoms but also the impact on fruit yield and quality and transmission efficiency through seeds, sap, and soil. For instance, as observed in the CGMMV-infected zucchini and ZGMMV-infected cucumber plants (Fig. 4d), less severe symptoms during cultivation could potentially allow infected plants to grow unnoticed. This could lead to the production and distribution of contaminated seeds, and subsequently, the transmission of these contaminants to other crops, resulting in severe spread of disease. Therefore, more comprehensive information is needed to more accurately assess the risk posed by tobamoviruses.

Despite significant differences in symptom severity and CP accumulation, the three viruses were found to infect all four cucurbit crops systemically. This suggests that a host plant can be infected by multiple viruses, a condition that is a prerequisite for recombination between two viruses, a key factor in virus evolution. Interestingly, a study by He et al. (2023) reported that an isolate of CGMMV, collected in Japan in 1971, carried partial segments with a high sequence identity to ZGMMV in the ORF of replication proteins. This suggests that interspecies recombination events between CGMMV and ZGMMV likely occurred in Japan. Recently, two new cucurbit-infecting tobamoviruses have been identified: the watermelon green mottle mosaic virus in Taiwan and California (Cheng et al. 2019, Pitman et al. 2019), and the Trichosanthes mottle mosaic virus in China (Chen et al. 2022). With the increase in international seed trade and the number of virus species, the coexistence of multiple seed-transmitted viruses in the same region is likely to become more common. This could potentially accelerate the evolution of these viruses.

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Authors' contributions

All authors contributed to the conception and design of the study. Material preparation was performed by Kenji Kubota and Yosuke Matsushita. K. Kubota performed data collection and analysis. All authors contributed to writing the first draft of the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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