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A Multiplex RT-PCR Assay for the Detection of Nine Virus Species Infecting Cucumber in Japan

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Abstract

A multiplex one-step reverse transcription polymerase chain reaction (multiplex RT-PCR) assay was developed to detect nine important viruses that infect cucumber in Japan: beet pseudoyellows virus (BPYV), cucurbit chlorotic yellows virus (CCYV), cucumber mosaic virus (CMV), kyuri green mottle mosaic virus (KGMMV), melon yellow spot virus (MYSV), papaya ringspot virus (PRSV), watermelon mosaic virus (WMV), watermelon silver mottle virus (WSMoV), and zucchini yellow mosaic virus (ZYMV). We newly designed virus species-specific primer pairs for seven viruses and used previously reported primer pairs for two viruses. Specificity and sensitivity tests by simplex RT-PCR using the primer set showed that touch-down RT-PCR effectively reduced non-specific amplification and had high sensitivity (10 - 10^5-fold dilution). For multiplex RT-PCR, the primer set was first divided into two sets: primer set I was for WMV, CCYV, KGMMV, BPYV, and WSMoV; primer set II was for PRSV, ZYMV, CMV, and MYSV. After optimizing the ratio of nine primer pairs and the number of cycles of the multiplex RT-PCR, all viruses could be detected by the same PCR condition. When the assays were applied to cucumber samples obtained from an open field and greenhouses, viral infections were clearly identified without non-specific amplification. Therefore, the multiplex RT-PCR assay can be used for the routine diagnosis of the nine viruses in field-growing samples.

Discipline: Agricultural Environment
Additional key words: *Cucumis sativus*, Cucurbitaceae, diagnosis, touch-down RT-PCR

Introduction

Cucurbitaceae includes several important edible crops worldwide. Among them, cucumber (*Cucumis sativus* L.) is a major crop (FAO 2023). Controlling plant viruses that cause yield loss is essential to produce these crops. At least 60 viruses have been reported to affect cucurbit crops, many of which also infect cucumber (Lecoq & Desbiez 2012, Gyoutoku et al. 2009). In Japan, cucumber accounts for approximately 2.2% of the total vegetable crop area (MAFF 2021). The following ten viruses are known to cause diseases such as mosaic, yellowing, and necrosis in cucumber leaves and fruits in Japan, which have caused problems such as reduced yield owing to the removal of infected plants, death, and reduced fruit quality. Beet pseudoyellows virus (BPYV) and cucurbit chlorotic yellows virus (CCYV) belong to the genus *Crinivirus* and are transmitted by whiteflies (Tzanetakis et al. 2013). Kyuri green mottle mosaic virus (KGMMV) belongs to the genus *Tobamovirus*. It is spread by soil contamination, foliage contact, and seeds (Yamamoto 1984, Tan et al. 2000). Cucumber mottle virus (CuMoV) also belongs to the genus *Tobamovirus* and has been confirmed to be transmitted through foliage contact (Orita et al. 2007). Melon yellow spot virus (MYSV) and watermelon silver mottle virus (WSMoV) belong to the genus *Orthotospovirus* and are transmitted by *Thrips palmi* Karny (Thysanoptera: Thripidae) (Riley et al. 2011). Cucumber mosaic virus (CMV) belongs to the genus *Cucumovirus*. In contrast, papaya ringspot virus (PRSV), watermelon mosaic virus (WMV), and zucchini yellow mosaic virus (ZYMV) belong to the genus *Potyvirus* and are mainly transmitted by aphids (Yamamoto 1986, Desbiez & Lecoq 1997,

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Jacquemond 2012, Chalak et al. 2017). Thus, vector organisms and modes of transmission differ greatly among viral species.

To prevent the spread of viral diseases, it is essential to identify the pathogenic virus species and implement control measures corresponding to the vector organism or mode of transmission. Enzyme-linked immunosorbent assay (ELISA), dot immunobinding assay, reverse transcription polymerase chain reaction (RT-PCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) are diagnostic techniques for viral diseases (Ohki 2009). Among these, RT-PCR is widely used because of its high detection sensitivity and low risk of contamination (Shirato 2019).

Multiplex RT-PCR assays have been developed to discriminate three (Shimomoto & Takeuchi 2006, Kuwabara 2009), four (Kwon et al. 2014), seven (Okuda et al. 2007), and eight (Okada et al. 2016) virus species infecting cucumber in Japan. However, these primer sets do not target CuMoV or WSMoV. Recently, diseases caused by WSMoV, which have long been known to occur in Okinawa Prefecture, located south of the Kyushu region (Iwaki et al. 1984, Okuda et al. 2001), have been reported in Kanagawa Prefecture, located in the Kanto region (Shimada et al. 2019), raising concerns about the spread of the disease to other regions. In this study, we developed a one-step multiplex RT-PCR assay for the simultaneous detection of nine viruses (BPYV, CCYV, CMV, KGMMV, MYSV, PRSV, WMV, WSMoV, and ZYMV) reported in cucumber in Japan, excluding CuMoV, the occurrence of which is extremely limited.

Materials and methods

1. Virus sources and RNA preparations

The viral isolates tested in the present study are listed in Table 1. To prepare cucumber plants infected with each virus (WMV, PRSV, ZYMV, CMV, KGMMV, and MYSV), we used W-80, PR-KK, E, 42CM, Y-1, and MY-Sz isolates, respectively. Freeze-dried leaves of plants infected with each virus were ground with 50 mM phosphate buffer (pH = 7.0) mixed with 10 mM sodium sulphite using a mortar and pestle. They were then mechanically inoculated onto the fully expanded cotyledons of *C. sativus* cv. Natsusuzumi using the carborundum method.

For WSMoV, diseased leaves of *Nicotiana benthamiana* Domin infected with OP and WD1 isolates were used as the inoculum source. Because the inoculation efficiency of WSMoV was low when 50 mM phosphate buffer with 10 mM sodium sulphite was used, 1 mM L-cysteine was added, and infected plants were produced using the same procedure. The OP isolate was inoculated into *Capsicum annuum* L. Grossum group cv. Almighty and was used for all the RT-PCR assays described below. The WD1 isolate was inoculated into *C. sativus* cv. Natsusuzumi and was used to evaluate primer set specificity.

As CCYV (Kurume) and BPYV (Saitama_Kumagaya_2018) do not cause infection by mechanical inoculation, the following procedure was used to create infected plants. CCYV-infected and healthy *C. sativus* cv. Natsusuzumi were placed in the same plastic cage (25 cm long × 30 cm wide × 28 cm high), and *Bemisia tabaci* (Gennadius) (biotype unknown) was released into

Table 1. Viral isolates used in this study

Virus	Isolate[a]	Original host	Geographical origin[b]	MAFF[c]
WMV	W-80	*Sicyos angulatus*	Kagawa	104039
PRSV	PR-KK	*Cucumis sativus*	Kyoto	260091
ZYMV	E	*C. sativus*	Ehime	260027
CCYV	Kurume	*C. sativus*	Fukuoka	-
CMV	42CM	*C. sativus*	Kagawa	104087
KGMMV	Y-1	*C. sativus*	Tokushima	104024
MYSV	MY-Sz	*C. melo*	Shizuoka	260128
BPYV	Saitama_Kumagaya_2018	*C. sativus*	Saitama	-
WSMoV	OP	*Capsicum annuum*	Okinawa	-
WSMoV	WD1	*Citrullus lanatus*	Okinawa	-

[a] Isolates W-80, PR-KK, E, 42CM, Y-1, and MY-Sz were supplied by the GeneBank Project, NARO (Tsukuba, Japan).
[b] Prefectural sites in Japan
[c] MAFF accession numbers of deposited viruses

the cage to transmit the virus. Similarly, the BPYV-infected and healthy *C. sativus* cv. Natsusuzumi plants were placed in a plastic cage, and *Trialeurodes vaporariorum* Westwood was released into the cage to transmit the virus and produce infected plants.

Total RNA was extracted from each virus's diseased leaves (approximately 10 mg) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). It was then diluted in distilled water to a concentration of 5 ng/μL and used for RT-PCR assays as described below. Total RNA was also extracted from healthy *C. sativus* cv. Natsusuzumi leaves using the same procedure, diluted to 5 ng/μL, and used as a negative control.

2. Primer design

The complete sequences of BPYV, CCYV, CMV, WSMoV, KGMMV, PRSV, and WMV isolates were obtained from the International Nucleotide Sequence Database (DDBJ/ENA/GenBank) (Table 2). The sequences of each viral isolate were aligned using ClustalW in MEGA X (Kumar et al. 2018), and regions of high homology between isolates within the virus species were visually selected. The region of the heat shock protein 70 homolog of BPYV and CCYV, 2a protein of CMV, RNA replicase of KGMMV, coat protein of PRSV and WMV, and glycoprotein precursor of WSMoV were selected to design the primers. Based on the sequences of these gene regions, specific primers for each virus were designed using Primer-BLAST from the National Center for Biotechnology Information (NCBI), setting the melting temperature (Tm) from 57°C to 63°C and the primer lengths from 19 nt to 25 nt (Table 3). For WMV, PRSV, and CMV, highly specific regions were selected based on primer-BLAST results, and degenerate primers were designed visually. For ZYMV and MYSV, specific primers designed by Okuda et al. (2007) were used (Table 3).

3. Determination of simplex RT-PCR conditions

Simplex RT-PCR was performed using the PrimeScript™ II High Fidelity One-Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan). The RT-PCR mixture included 6.25 μL of 2 × One-Step High Fidelity Buffer, 0.25 μL of PrimeScript II RT Enzyme Mix, 1 μL of PrimeSTAR GXL for One-Step RT-PCR, 0.5 μL of each of the 0.4 μM primers, and 2 μL of 5 ng/μL total RNA template, and the final volume was adjusted to 12.5 μL with distilled water. The RT-PCR conditions were as follows: reverse transcription at 45°C for 10 min and denaturation at 94°C for 2 min to inactivate the reverse transcriptase, followed by 30 cycles for denaturation at 98°C for 10 s, annealing at 55°C for 15 s, and elongation at 68°C for 12 s. An S1000 thermal cycler (Bio-Rad Laboratories, Inc.) was used for this reaction. Amplified products were observed under UV irradiation after electrophoresis on a 2.5% agarose gel and stained with ethidium bromide.

For the virus and primer combinations in which non-specific amplification was observed, the annealing conditions were as follows: 55°C for 30 cycles (55°C × 30); 60°C for 5 cycles with a 1°C reduction for each cycle, followed by 25 cycles at 55°C (60°C − 1°C × 5/55°C × 25); 65°C for 10 cycles with a 1°C reduction for each cycle followed by 20 cycles at 55°C (65°C – 1°C × 10/55°C × 20). After RT-PCR and touch-down RT-PCR, the amplified products were confirmed by agarose gel electrophoresis, as described above.

Table 2. Viral sequence used for the design of the primers in this study

Virus	Accession no.[a]
BPYV	AY330919, LC100132
CCYV	JN126045-JN126046, JN641883, JF502222, JQ904628-JQ904629, KU507601-KU507602, KY400632-KY400637, KY618798-KY618799, MH477611-MH477612
CMV	AB079889-AB079890, AB188228, AB188231-AB188232, AB188234, AB188229, AB188235, AF033667, AF071551, AF314188, EU665000-EU665001, HE962478-HE962479, KX883763-KX883764, LC381763-LC381764, LC390004-LC390005, LC390165-LC390166
KGMMV	AB015145, AB162006, AJ295948
PRSV	AY027810, DQ374152-DQ374153, JN132408-JN132444, JN132446-JN132465, JN132467-JN132471, MH427286
WMV	AB218280, AB369278, DQ399708, EU660578-EU660590, FJ823122, HQ384216, JF273458-JF273463, JF273468-JF273469, JX079685, KC292915, KF274031, KM597070-KM597071, KP100058, KT992068-KT992093, KU240094-KU240110, KU246036, KX512320, KX664483, KX926428, LC412927, MG194418, MH469650, MK217416, NC_006262
WSMoV	AB042649-AB042650, AF133128, AY863200, AY864852, DQ157768, KM242056, JX177645-JX177647, NC_003841, NC_003843, Z46419

[a] The serial accession no. is connected by a hyphen.

Table 3. Specific primer pairs designed for one-step reverse transcription-polymerase chain reaction

Primer set[a]	Target virus	Name[b]	Sequence (5′ to 3′)	Length (nt)	Size (bp)[c]	Reference
I	WMV	WMV-poly-F3	TCTGARAGTCCRTATATGCCTAGAT	25	222	This study
		WMV-poly-R3	RTGCCTYTCAGTATTTTCGGAATT	24		
	CCYV	CCYV-HSP-F7	TTCTATGTCACACGCTCAAGAGG	23	514	This study
		CCYV-HSP-R7	AATCGTGGGTGTTTTCACGTAAG	23		
	KGMMV	KG-rep-F1	CTGGACACACAAGACAAACGC	21	616	This study
		KG-rep-R1	CAGCGAAGAGAACCTTGATGTTT	23		
	BPYV	BP-HSP-F1	TGAACTTTTCACTCCGGAGAAT	22	1048	This study
		BP-HSP-R1	TCGAAAGCTGACAAGATTGAAA	22		
	WSMoV	WS-M-F2	AGTATTTTCAACAGAGGTTGATGG	24	1155	This study
		WS-M-R2	TCTTGTTGATCTTCAGGGATGAGT	24		
II	PRSV	PRSV-poly-F13	GGTGTYTGGGTKATGATGGATGGG	24	323	This study
		PRSV-poly-R13	CCGTCCATWCCAAACATTCTGCGA	24		
	ZYMV	mu-ZY4F	GGATAAATTGATGAGAGCATTA	22	410	Okuda et al. (2007)
		mu-ZY4R	TGTCAAGTAAGCCGCTATC	19		
	CMV	CMV-2a-F1	CAACATGGAAGCTAAGGTGATG	22	576	This study
		CMV-2a-R2	GCYCTCTCGCTGGGACTTTTGT	22		
	MYSV	mu-MY4F	CATTCTGTTGTTTGATGGAAC	21	888	Okuda et al. (2007)
		mu-MY4R	TCCTAAGTAAACACCATGTCTAC	23		

[a] Primer set is divided into I and II for multiplex RT-PCR.
[b] The upper and lower columns correspond to primers for the sense and complementary strands.
[c] Predicted sizes of amplified fragments

4. Specificity and sensitivity test of the primer set

To evaluate primer specificity, touch-down RT-PCR was performed with annealing conditions of 65°C – 1°C × 10/55°C × 20 for all combinations of virus and primer pairs. The amplified products were confirmed by agarose gel electrophoresis, as described above. The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the Eurofins Genomics contract service. Sequence data were searched using Nucleotide BLAST at NCBI to confirm the target viral sequence.

The detection limits of each virus were determined by making 10-fold serial dilutions (10^{0}-10^{-5}) of the extracted total RNAs diluted with distilled water. Touch-down RT-PCR was performed using the target primers. The amplified products were confirmed by agarose gel electrophoresis, as described above.

5. Determination of multiplex RT-PCR conditions

Multiplex RT-PCR was optimized to enable the synchronous amplification of nine target regions. Total RNA from nine viruses was mixed to RNA concentrations of 5 ng/μL each and used as a template for multiplex RT-PCR. Based on the results of the sensitivity test, the primer set was divided into two sets (Table 3): primer set I consisted of WMV-poly-F3/WMV-poly-R3, CCYV-HSP-F7/CCYV-HSP-R7, KG-rep-F1/KG-rep-R1, BP-HSP-F1/BP-HSP-R1, and WS-M-F2/WS-M-R2; primer set II consisted of PRSV-poly-F13/PRSV-poly-R13, mu-ZY4F/mu-ZY4R, CMV-2a-F1/CMV-2a-R2, and mu-MY4F/mu-MY4R.

The optimal factor for multiplex RT-PCR was the ratio of the nine pairs of primers and the number of cycles. The first step was to examine primer concentrations with a fixed number of cycles: the annealing temperature and cycle number were 65°C for 10 cycles with 1°C reduction for each cycle followed by 25 cycles at 55°C (65°C – 1°C × 10/55°C × 25) in primer set I-V1 to V4, and 65°C – 1°C × 10/55°C × 20 in primer set II-V1 to V3 (Tables 6, 7). Then, the number of cycles was compared in the following two treatments: 65°C – 1°C × 10/55°C × 20 and 65°C – 1°C × 10/55°C × 25. The primers were added to the RT-PCR mixture at 1 μL in total, and other conditions were the same as described above. The amplified products were confirmed by agarose gel electrophoresis, as described above.

6. Application of simplex and multiplex RT-PCR

To clarify the practicality of the RT-PCR assay developed in this study, viral infection was surveyed in

Table 4. Symptoms of field samples collected in Miyazaki and Kumamoto prefectures

Sample	Location	Variety[a]	Symptom
K1	Miyazaki City, Miyazaki Prefecture	-	yellow spots
K2		-	yellow spots
K3	Kumamoto City, Kumamoto Prefecture	-	necrosis
K4		-	necrosis
K5		-	yellowing
K6	Koshi City, Kumamoto Prefecture	Courage	chlorotic spots
K7		Courage	yellow spots
K8		Courage	chlorotic yellows
K9		Tokiwa kazemidori	mosaic
K10		Tokiwa kazemidori	mosaic

[a] The hyphen indicates unknown.

cucumbers cultivated in an open field or greenhouses. Cucumber leaves showing virus-like symptoms were collected from a greenhouse in Miyazaki, Miyazaki Prefecture (K1-K2), one greenhouse in Kumamoto, Kumamoto Prefecture (K3-K5), and one greenhouse (K6-K8) and an open field (K9-K10) in Koshi, Kumamoto Prefecture, and these ten samples were stored at −80°C. Leaf symptoms of the collected leaves are shown in Table 4.

Total RNA was extracted from the collected leaves using the RNeasy Plant Mini Kit (Qiagen) and used as a template. Simplex and multiplex RT-PCR were performed with the annealing conditions of 65°C – 1 °C × 10/55°C × 20 and 65°C – 1°C × 10/55°C × 25, respectively. The amplified products were confirmed by agarose gel electrophoresis, as described above.

Results

1. Determination of simplex RT-PCR conditions

To determine the optimal conditions for simplex RT-PCR, the annealing temperatures were investigated for all combinations of template RNA and each primer pair. First, RT-PCR was performed at an annealing temperature of 55°C for 30 cycles, and amplified products of the expected size were confirmed for each primer pair in combination with the targeted virus. Non-specific amplification products were observed when the PRSV-poly-F13/PRSV-poly-R13 primers were used for ZYMV, KGMMV, and WSMoV at more than 1,200 bp, about 600 bp, and 700 bp, respectively. Similarly, the mu-ZY4F/mu-ZY4R and CMV-2a-F1/CMV-2a-R2 primers amplified about 1,500 bp each in combination with PRSV, and the WS-M-F2/WS-M-R2 primers amplified over 2,500 bp in combination with KGMMV as non-specific amplifications.

To suppress non-specific amplification, touch-down RT-PCR was performed for combinations in which non-specific amplification was observed. When the annealing temperature was set to 60°C – 1°C × 5/55°C × 25, non-specific amplification disappeared in the combination of PRSV-poly-F13/PRSV-poly-R13 primers and WSMoV, mu-ZY4F/mu-ZY4R primers and PRSV, CMV-2a-F1/CMV-2a-R2 primers and PRSV. Non-specific amplification was observed with combinations of PRSV-poly-F13/PRSV-poly-R13 primers and ZYMV, PRSV-poly-F13/PRSV-poly-R13 primers and KGMMV, and WS-M-F2/WS-M-R2 primers and KGMMV. When the annealing temperature was set to 65°C – 1°C × 10/55°C × 20, no non-specific amplification was observed in any combination.

2. Specificity and sensitivity test of the primer set

To confirm the specificity of the primer pairs, touch-down RT-PCR was performed with all combinations of template RNA and each primer pair, with an annealing temperature of 65°C – 1°C × 10/55°C × 20. Amplified products of the expected sizes were observed only in combination with the target viruses using each primer pair (Fig. 1). In addition, non-specific amplification products were not observed for any combination (Fig. 1). Template RNA for WSMoV was extracted from *C. annuum*. Similar results were obtained for template RNA extracted from *C. sativus* (Fig. 2).

The amplified products were sequenced directly and deposited in DDBJ/ENA/GenBank (Accession nos.: LC778418–LC778426; Table 5). Among them, seven viruses, WMV, PRSV, CCYV, CMV, KGMMV, MYSV, and BPYV, showed more than 99% homology with sequences of the target viruses in the database (Accession nos.: MW483119 [WMV], AB583216 [PRSV], KY400634 [CCYV], AB368497 [CMV], AB015145 [KGMMV],

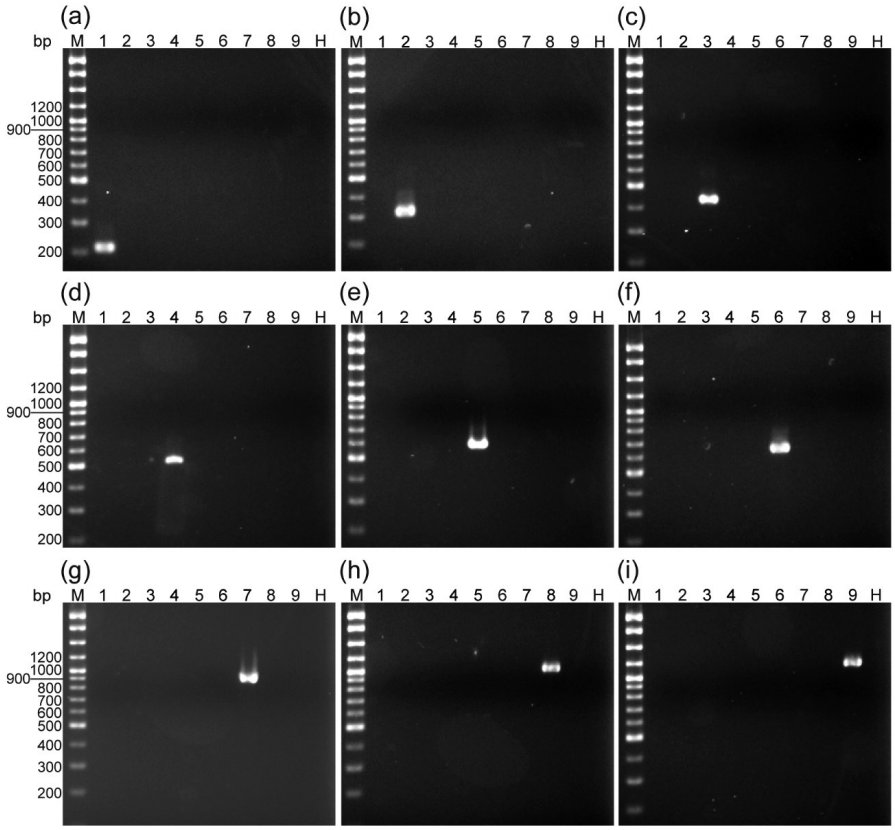

Fig. 1. Agarose gel electrophoresis of products obtained from touch-down RT-PCR using WMV-poly-F3/WMV-poly-R3 primer (a), PRSV-poly-F13/PRSV-poly-R13 primer (b), mu-ZY4F/mu-ZY4R primer (c), CCYV-HSP-F7/CCYV-HSP-R7 primer (d), CMV-2a-F1/CMV-2a-R2 primer (e), KG-rep-F1/KG-rep-R1 primer (f), mu-MY4F/mu-MY4R primer (g), BP-HSP-F1/BP-HSP-R1 primer (h), and WS-M-F2/WS-M-R2 primer (i)

Touch-down RT-PCR was performed using the total RNA extracted from plants infected with WMV (lane 1), PRSV (lane 2), ZYMV (lane 3), CCYV (lane 4), CMV (lane 5), KGMMV (lane 6), MYSV (lane 7), BPYV (lane 8), and WSMoV (lane 9). Lane H, total RNA extracted from healthy cucumber plants. Lane M, 100 bp ladder markers (Norgen, Canada).

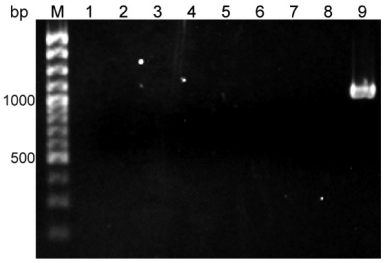

Fig. 2. Specificity evaluation of each primer pair using the total RNA extracted from cucumber infected with WSMoV

Touch-down RT-PCR was performed using WMV-poly-F3/WMV-poly-R3 primer (lane 1), PRSV-poly-F13/PRSV-poly-R13 primer (lane 2), mu-ZY4F/mu-ZY4R primer (lane 3), CCYV-HSP-F7/CCYV-HSP-R7 primer (lane 4), CMV-2a-F1/CMV-2a-R2 primer (lane 5), KG-rep-F1/KG-rep-R1 primer (lane 6), mu-MY4F/mu-MY4R primer (lane 7), BP-HSP-F1/BP-HSP-R1 primer (lane 8), and WS-M-F2/WS-M-R2 primer (lane 9). Lane M, 100-bp ladder markers (Norgen, Canada).

Table 5. Sequence results of the amplified products using simplex RT-PCR

Target virus	Viral isolate	Length (nt)[a]	Accession no.
WMV	W-80	109	LC778418
PRSV	PR-KK	208	LC778419
ZYMV	E	312	LC778420
CCYV	Kurume	412	LC778421
CMV	42CM	439	LC778422
KGMMV	Y-1	490	LC778423
MYSV	MY-Sz	788	LC778424
BPYV	Saitama_Kumagaya_2018	776	LC778425
WSMoV	OP	674	LC778426

[a] The inaccurate parts of the nucleotide sequence were omitted.

AB038343 [MYSV], LC100132 [BPYV]). WSMoV and ZYMV showed 96% and 98% homology, respectively, with the target virus sequences in the database (Accession nos.: MW051789 [WSMoV] and MH042026 [ZYMV]).

The detection limits for simplex RT-PCR were determined using a 10-fold serial dilution (10^0-10^{-5}) of template RNAs. The results showed that the highest dilutions for electrophoretic detection were 10^{-1} for WMV and WSMoV (Fig. 3a, i); 10^{-2} for CCYV and BPYV (Fig. 3d, h); 10^{-3} for PRSV, ZYMV, and MYSV (Fig. 3b, c, g); 10^{-4} for CMV (Fig. 3e); and at least 10^{-5} for KGMMV (Fig. 3f).

3. Determination of multiplex RT-PCR conditions

The primer concentrations and number of cycles were examined to optimize the multiplex RT-PCR conditions. Five pairs of primers (primer set I) and four pairs of primers (primer set II) were evaluated in different proportions (Tables 6 and 7). The results of primer set I showed that only in the case of RT-PCR with V4, all expected amplification products were observed for each target virus alone and for the mixed RNA of the nine viruses (Table 6). When KGMMV alone was used as the template RNA, non-specific amplification products were observed at more than 1,500 bp for all versions of primer

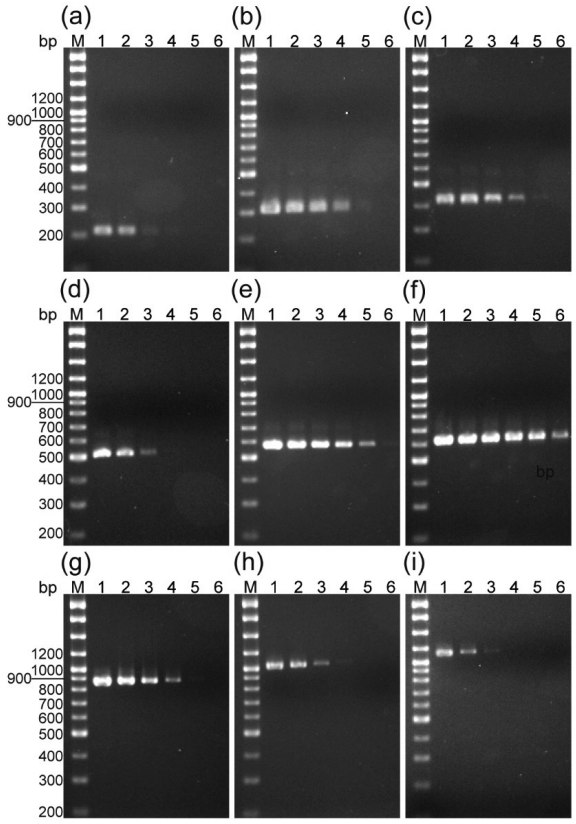

Fig. 3. Sensitivities of detection of WMV (a), PRSV (b), ZYMV (c), CCYV (d), CMV (e), KGMMV (f), MYSV (g), BPYV (h), and WSMoV (i), using the touch-down RT-PCR assay employing each specific primer set
Total RNA preparations from viral infected plants were adjusted to 5 ng/μL. Tenfold serial dilutions of each virus-infected RNA preparation in 10^0 (lane 1), 10^{-1} (lane 2), 10^{-2} (lane 3), 10^{-3} (lane 4), 10^{-4} (lane 5), and 10^{-5} (lane 6) were subjected to touch-down RT-PCR. Lane M, 100 bp ladder markers (Norgen, Canada).

Table 6. Results of multiplex RT-PCR using primer set I in each version

RNA	Target virus	Result[a]			
		V1	V2	V3	V4
WMV	WMV	+	+	+	+
CCYV	CCYV	+	+	+	+
KGMMV	KGMMV	−[b]	+[b]	+[b]	+[b]
BPYV	BPYV	+	+	+	+
WSMoV	WSMoV	+	+	+	+
Nine virus mix	WMV	+	+	+	+
	CCYV	+	+	+	+
	KGMMV	−	+	+	+
	BPYV	+	−	−	+
	WSMoV	+	+	+	+
Healthy cucumber	−	−	−	−	−

[a] +, positive results for the test; −, negative results for the test. Primer concentrations (μM) of WMV-poly-F3/WMV-poly-R3, CCYV-HSP-F7/CCYV-HSP-R7, KG-rep-F1/KG-rep-R1, BP-HSP-F1/BP-HSP-R1, and WS-M-F2/WS-M-R2 are 0.4, 0.2, 0.01, 0.8, and 0.4 in V1; 0.4, 0.2, 0.05, 0.8, and 0.4 in V2; 0.4, 0.2, 0.1, 0.8, and 0.4 in V3; 0.4, 0.2, 0.02, 0.8, and 0.2 in V4.
[b] Non-specific amplifications of more than 1,500 bp were confirmed.

Table 7. Results of multiplex RT-PCR using primer set II in each version

RNA	Target virus	Result[a]		
		V1	V2	V3
PRSV	PRSV	+	+	+
ZYMV	ZYMV	+	+	+
CMV	CMV	+	+	+
MYSV	MYSV	+	+	+
Nine virus mix	PRSV	+	+	+
	ZYMV	+	−	+
	CMV	+	+	+
	MYSV	−	−	+
Healthy cucumber	−	−	−	−

[a] +, positive results for the test; −, negative results for the test. Primer concentrations (μM) of PRSV-poly-F13/PRSV-poly-R13, mu-ZY4F/mu-ZY4R, CMV-2a-F1/CMV-2a-R2, and mu-MY4F/mu-MY4R are 0.4, 0.4, 0.4, and 0.4 in V1; 0.4, 0.8, 0.4, and 0.8 in V2; 0.2, 0.8, 0.2, and 0.8 in V3.

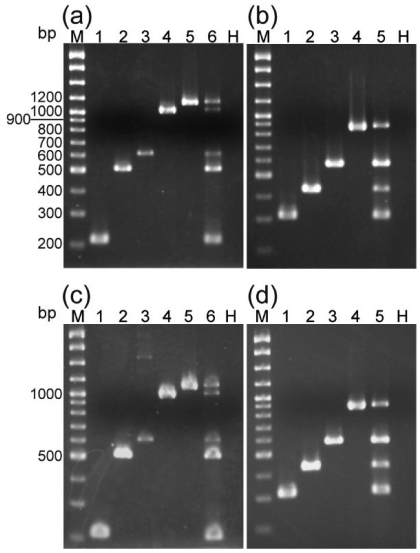

Fig. 4. Simultaneous detection for nine viruses by multiplex RT-PCR using primer set I-V4 (a, c) and primer set II-V3 (b, d) at 65℃ – 1℃ × 10/55℃ × 20 (a, b) and 65℃ – 1℃ × 10/55℃ × 25 (c, d)

In primer set I-V4 (a, c), of total RNA extracted from plants, WMV alone (lane 1), CCYV alone (lane 2), KGMMV alone (lane 3), BPYV alone (lane 4), WSMoV alone (lane 5), and a mix of nine viruses (lane 6) were subjected to multiplex RT-PCR. In primer set II-V3 (b, d), of total RNA extracted from plants, PRSV alone (lane 1), ZYMV alone (lane 2), CMV alone (lane 3), MYSV alone (lane 4), and a mix of nine viruses (lane 5) were subjected to multiplex RT-PCR. Lane H, total RNA extracted from healthy cucumber plants. Lane M, 100 bp ladder markers (Norgen, Canada).

set I (Table 6). In primer set II, all the expected amplification products were observed only when RT-PCR was performed with V3 for each target virus alone and for the mixed RNA of the nine viruses (Table 7). Non-specific amplification products were not observed for primer set II.

The number of cycles in multiplex RT-PCR was then examined using primer sets I-V4 and II-V3. When the number of cycles was 65°C – 1°C × 10/55°C × 20, all expected amplification products were observed for each target virus alone and for the mixed RNA of nine viruses, in both primer set I and primer set II (Fig. 4a, b). Similar amplified products were observed when the number of cycles was 65°C – 1°C × 10/55°C × 25 in both primer set I and primer set II (Fig. 4c, d). A non-specific amplification product above 1,500 bp was observed in the combination of KGMMV alone and primer set I only when the number of cycles was 65°C – 1°C × 10/55°C × 25 (Fig. 4c).

Based on a series of results, the multiplex RT-PCR procedures that were determined are shown in Table 8.

4. Application of simplex and multiplex RT-PCR

Ten cucumber leaf samples showing virus-like symptoms were collected from an open field and greenhouses in Miyazaki and Kumamoto Prefectures. All samples were assayed using simplex and multiplex RT-PCR. The simplex and multiplex RT-PCR results were consistent (Figs. 5, 6). CCYV and MYSV were detected in K1 and K2 with yellow spots, and in K3 and K4 with necrosis (Fig. 5d, g; Fig. 6a, b; Table 4). CCYV was detected in K5 with yellowing, K6 with chlorotic spots, K7 with yellow spots, and K8 with chlorotic yellows (Fig. 5d; Fig. 6a; Table 4). CCYV and WMV were detected in K9 and K10 with mosaic symptoms (Fig. 5a, d; Fig. 6a; Table 4). No non-specific amplification was observed in any of the samples tested.

Discussion

When a viral disease is suspected in cucumber, it is possible to identify the most likely virus species to some extent based on the area of occurrence, pests, and disease symptoms. However, RT-PCR is rapid and useful for reliably identifying the virus species. In this study, we developed a multiplex RT-PCR assay that can specifically detect nine viruses causing diseases in cucumbers in Japan.

To specifically detect the target virus by RT-PCR, it is essential to consider temperature conditions and other factors. The annealing temperature is related to generating non-specific amplification products (Liu et al. 2019). Touch-down RT-PCR increases the specificity and detection sensitivity of the amplified products by setting the initial annealing temperature higher than the Tm and gradually lowering it to the Tm (Korbie & Mattick 2008). In the present study, only touch-down RT-PCR with an annealing temperature of 65°C – 1 °C × 10/55°C × 20 successfully detected all target viruses without non-specific amplified products by simplex RT-PCR.

Multiplex RT-PCR is an effective tool for accelerating diagnosis and amplifying multiple RNA regions in the same tube by mixing multiple primer pairs. By designing each primer pair such that the amplification size of the target RNA region differs, the amplified products can be distinguished by electrophoresis. Okuda et al. (2007) reported a multiplex RT-PCR assay to detect eight viruses, including seven that are problematic in cucumber. However, the problem in this assay is that when KGMMV was present, other viruses could not be detected simultaneously. Most likely, this is because the virus concentration in infected plants is very high in KGMMV (Yamamoto 1984, Tan et al. 2000), which suppresses the amplification of other viruses. In this

Table 8. The procedure of multiplex RT-PCR developed in this study

A

Procedure	Detail explanation		
	Primer set	Primer pair	Concentration (μM)
Preparing primer sets[a]	I	WMV-poly-F3/WMV-poly-R3	0.4
		CCYV-HSP-F7/CCYV-HSP-R7	0.2
		KG-rep-F1/KG-rep-R1	0.02
		BP-HSP-F1/BP-HSP-R1	0.8
		WS-M-F2/WS-M-R2	0.2
	II	PRSV-poly-F13/PRSV-poly-R13	0.2
		mu-ZY4F/mu-ZY4R	0.8
		CMV-2a-F1/CMV-2a-R2	0.2
		mu-MY4F/mu-MY4R	0.8

B

Procedure	Detail explanation	
Multiplex RT-PCR using primer set I and II, respectively	Step 1	Reverse transcription at 45°C for 10 min
	Step 2	Denaturation at 94°C for 2 min
	Repeat following steps 3–5 for 10 cycles	
	Step 3	Denaturation at 98°C for 10 s
	Step 4	Annealing at 65°C for 15 s with 1°C reduction for each cycle
	Step 5	Elongation at 68°C for 12 s
	Repeat following steps 6–8 for 25 cycles[b]	
	Step 6	Denaturation at 98°C for 10 s
	Step 7	Annealing at 55°C for 15 s
	Step 8	Elongation at 68°C for 12 s
Observation of amplified products after electrophoresis	See "Materials and methods"	

[a] In simplex RT-PCR, all primer concentrations were adjusted to 0.4μM.
[b] In simplex RT-PCR, steps 6–8 were repeated for 20 cycles.

study, KGMMV was detectable even at 10^{-5} (100 fg per 12.5 μL reaction mixture), the lowest concentration in the study, suggesting a very high virus concentration in the total RNA. Okada et al. (2016) modified the assay of Okuda et al. (2007) and developed a multiplex RT-PCR assay to detect nine viruses with the addition of CCYV. However, this has yet to solve the problem of mixed KGMMV infection. In the present study, the concentrations of primer pairs were adjusted with reference to the detection limit of each virus, and simultaneous detection of KGMMV and other viruses by multiplex RT-PCR was successfully achieved. In addition, we included WSMoV as a target for detection, which has not been included in previous studies. WSMoV was first identified in Japan in 1982 in Okinawa Prefecture, and thereafter, its occurrence was limited to Kagoshima and Okinawa Prefectures (Iwaki et al. 1984, Okuda 2016). However, in 2016, WSMoV infection in cucumber was confirmed in Kanagawa Prefecture, Honshu, raising concerns about the spread of the disease in the future (Shimada et al. 2019). Therefore, the RT-PCR assay developed in this study is useful for routine surveys of cucumber viral diseases and preventing the spread of WSMoV in Japan.

Multiplex RT-PCR has often been reported to have inferior detection sensitivity compared with simplex RT-PCR (Kumar et al. 2017, Liu et al. 2019). We then confirmed the result of multiplex RT-PCR with an increased number of cycles, 65°C – 1°C × 10/55°C × 25, to improve detection sensitivity, and observed clear amplified products. However, a non-specific amplification was observed in the combination of KGMMV alone and primer set I-V4. As non-specific amplification was observed above 1,500 bp, it could be distinguished from other viral species by electrophoresis and was not considered a practical problem.

In applying simplex and multiplex RT-PCR to ten cucumber samples, WMV and MYSV were detected in

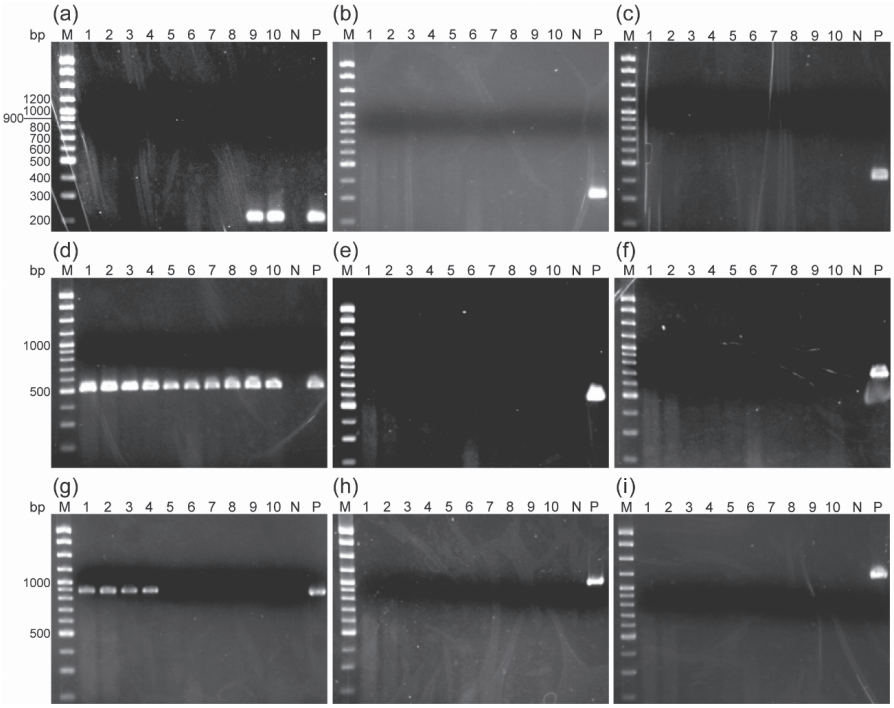

Fig. 5. Detection of viruses from field samples by simplex RT-PCR using primer sets for WMV (a), PRSV (b), ZYMV (c), CCYV (d), CMV (e), KGMMV (f), MYSV (g), BPYV (h), and WSMoV (i)

The RT-PCR was performed using the total RNAs extracted from cucumber samples. These samples were collected from Miyazaki City, Miyazaki Prefecture (lanes 1–2 represent K1–K2); Kumamoto City, Kumamoto Prefecture (lanes 3–5 represent K3–K5); Koshi City, Kumamoto Prefecture (lanes 6–10 represent K6–K10). Lane N, total RNA extracted from healthy cucumber plants. Lane P, total RNAs extracted from plants infected with WMV (a), PRSV (b), ZYMV (c), CCYV (d), CMV (e), KGMMV (f), MYSV (g), BPYV (h), and WSMoV (i). Lane M, 100 bp ladder markers (Norgen, Canada).

some samples, and CCYV was detected in all samples, and these results were consistent with both assays. The samples in which WMV and MYSV were detected showed the characteristic symptoms of each disease, consistent with the diagnostic results. However, the characteristic symptoms of CCYV, such as chlorotic spots, yellow spots, and yellowing (Gyoutoku et al. 2009, Kuwabara et al. 2009), were not confirmed in some samples, although CCYV was detected. This is because the samples that showed no symptoms of CCYV were mixed infections with other viruses, suggesting that only one of the viruses was pathogenic at the time of collection, and CCYV had reached a concentration detectable by RT-PCR before it caused the symptoms of CCYV. Thus, the present assays enable the rapid and accurate detection of viruses that cannot be determined by disease symptoms alone, contributing to preventing the spread of viral diseases.

In conclusion, the multiplex RT-PCR assay developed in this study is applicable for diagnosing WSMoV, which has recently become a growing concern. The assay can detect almost all viruses reported in cucumbers in Japan. In addition, the problem of missed detection owing to mixed KGMMV infections reported in previous studies was resolved using the present multiplex RT-PCR assay. The multiplex RT-PCR assay is a powerful tool for viral monitoring and routine viral diagnostics to reduce detection costs and time.

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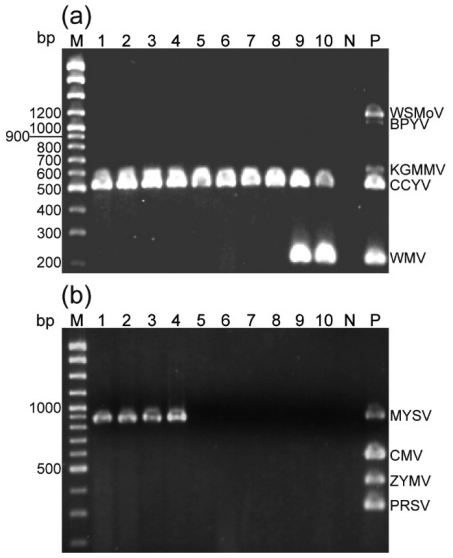

Fig. 6. Detection of viruses from field samples by multiplex RT-PCR using primer set I-V4 (a) and primer set II-V3 (b)

RT-PCR was performed using the total RNAs extracted from cucumber samples. These samples were collected from Miyazaki City, Miyazaki Prefecture (lanes 1–2 represent K1–K2); Kumamoto City, Kumamoto Prefecture (lanes 3–5 represent K3–K5); and Koshi City, Kumamoto Prefecture (lanes 6–10 represent K6–K10). Lane N, total RNA extracted from healthy cucumber plants. Lane P, a mix of total RNA extracted from cucumber samples infected with each of the nine target viruses. Lane M, 100 bp ladder markers (Norgen, Canada).

providing the field samples showing virus-like symptoms. We are grateful to M. Okuda, N. Mizutani, T. Kitamura, and S. Tanaka for their valuable comments. We are grateful to E. Abe, M. Nagata, and K. Sato for their kind support.

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