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Application of Multiplex RT-PCR for Detection of Cucurbit-infecting Tobamovirus

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Abstract

Cucumber green mottle mosaic virus (CGMMV) and *Kyuri green mottle mosaic virus* (KGMMV) are seed borne viruses and they are also transmitted mechanically during agricultural practice and through water. Hence, these viruses have potential diseases widely distributed throughout the world. To detect different strains of CGMMV and KGMMV, several specific primers for each virus were designed for single and multiplex RT-PCR. The results of single and multiplex RT-PCR showed that CGMMV was detected in zucchini isolated in Bali-Indonesia, while KGMMV was detected both in zucchini isolated in Bali-Indonesia and *Cucumis metuliferus* isolated in Thailand. Furthermore, artificial co-infection of these two viruses was prepared and carried out using two different ways of viral RNAs extraction. Based on the results, it could be reported that viral RNAs for cDNA amplification by multiplex RT-PCR could be extracted from a mixture of infected leaves or separate extraction of each viruses infected leaves. In addition, results presented in this study demonstrated the application of multiplex RT-PCR to simultaneously detect CGMMV and KGMMV from cucurbit leaves using a mixture of four primers and its feasibility as a sensitive and rapid laboratory assay. Since, no multiplex RT-PCR technique has been described for the detection of CGMMV and KGMMV, this technique can be a good option for sensitive and reliable tool for detection of two major cucurbit infecting Tobamoviruses.

Keywords : Cucurbit infecting Tobamovirus, multiplex RT-PCR, seed borne viruses

Introduction

In plant diagnostic work, the use of molecular techniques has increased in recent years. Molecular techniques such as reverse transcription and polymerase chain reaction (RT-PCR) detection enable a sensitive detection of plant RNA viruses and are necessary for certification programs. However, most of these techniques can detect only one pathogen for one test, while different plant pathogens may occur at the same time. The two of important cucurbit-infecting Tobamovirus, *Cucumber green mottle mosaic virus* (CGMMV) and *Kyuri green mottle mosaic virus* (KGMMV) cause economical losses in some countries (Ugaki *et al.*, 1991; Tan *et al.*, 2000). Strains of CGMMV were first reported from the United Kingdom and Europe by Ainworth in 1935, while KGMMV was first found in cucumber cultivated in Tokushima prefecture, Japan in 1966 (Inoue, 1967). Furthermore, KGMMV was presently reported also in Korea (Lee *et al.*, 2000) and Indonesia (Daryono, *et al.*, 2005; 2006). CGMMV and KGMMV are soil and seed borne virus, transmitted mechanically, and through water (Blancard *et al.*, 1994; Tan *et al.*, 2000). As they have similar host range and symptom, the development of sensitive methods for their detection is pivotal for diagnosis of viruses in cucurbit plants. Up to

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now, the presence of CGMMV and KGMMV in cucurbit plants has been tested mainly by enzyme-linked immunosorbent assay (ELISA).

During recent years, molecular based techniques such as RT-PCR have been developed enabling the direct detection of cucurbit viruses such as *Cucumber mosaic virus* (CMV) (De Blas *et al.*, 1994), and *Zucchini yellow mosaic virus* (ZYMV) (Barbara *et al.*, 1995; Thompson *et al.*, 1995). Some members of Tobamovirus such as *Pepper mild mottle virus* (PMMoV; Tenllado *et al.*, 1994) and *Tobacco mosaic virus* (TMV; Drygin *et al.*, 1992) can be detected by RT-PCR for reducing the time duration of experiments. However, there is no report or procedures to detect CGMMV and KGMMV in one step reaction.

The objective of this study was to compare the single and multiplex RT-PCR for the simultaneous detection of CGMMV and KGMMV in melon and other cucurbit plants.

Materials and Methods

Virus sources

Two isolates of Indonesian KGMMV (KGMMV-YM and KGMMV-YL) were isolated from leaves showing mottle mosaic symptom of zucchini plants in Bali-Indonesia (BIZ) and Vietnam (VT), and from samples of *Cucumis metuliferus* collected in Thailand (TH). The samples were stored at -80°C before used for RNA extraction. Watermelon strain of CGMMV (CGMMV-W) and a cucumber strain of KGMMV (KGMMV-C) samples were provided by the Japan Plant Protection Association, Tsukuba- Japan to be used as positive control.

RNA extraction

Viral RNAs were extracted from samples according to the method described by Rosner *et al.* (1983). To study co-infection of CGMMV and KGMMV, mRT-PCR was employed using two different RNA extractions (Figure 1). In the first experiment (I), viral RNAs were extracted from a mixture of infected leaf samples of CGMMV-W and KGMMV-C. The obtained RNAs were used synthetic cDNA. In the second experiment (II), viral RNAs were extracted separately from each infected leaf samples of CGMMV and KGMMV and these RNAs were used to obtain cDNA.

Design of primers

In order to detect CGMMV and KGMMV in one step reaction, primarily two pairs of primers were designed (Table 1). The first set

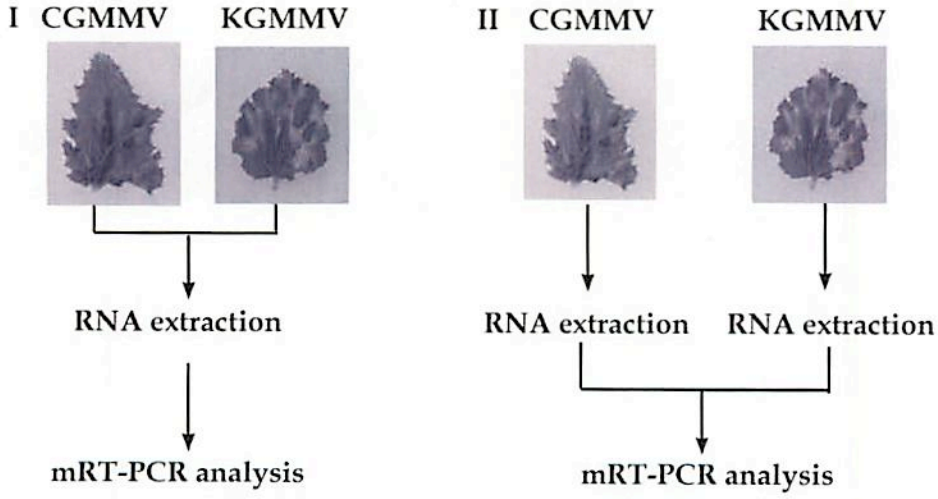

Figure 1. Flow chart of two RNA extractions method. I: RNAs were obtained by mixed CGMMV and KGMMV infected leaves; II: RNAs were obtained by mixed each of RNAs CGMMV and KGMMV

Table 1. List of primers used for single and multiplex RT-PCR detection of CGMMV and KGMMV

Primers	Sequence 5′ 3′	Genome position	Size (bp)	Detection
CG-F	ACGTACCGGAATCCTGTAGGGGT	5238-5260	697	CGMMV
CG-R	TCTACGACAGASGAGGGTAACGC	5913-5935		
KG-F	GGGTTGTTAACYTCTGTTGCGTC	6016-6038	335	KGMMV
KG-R	TCADTTWGAGGAAGWNGKGCTTG	6329-6351		

of primers (CG-F and CG-R) were designed to detect CGMMV strains specifically and constructed based on the partial sequences of coat and movement protein genes of eight CGMMV strains (CGMMV-Y: AJ245440; CGMMV-W: AB015146; CGMMV-SH: D12505; CGMMV-NS: AJ243831; CGMMV-KW: AF417242; CGMMV-KOM: AF417243; CGMMV-India: AY309021; CGMMV-GR7: AJ459423). The second set of primers (KG-F and KG-R) were designed to detect KGMMV strains specifically and constructed based on the coat protein gene sequences of four KGMMV strains (KGMMV-YM: AB162006; KGMMV-Y:AB015145; KGMMV-C: AB015144; KGMMV-C1: AJ295948).

Reverse transcription and polymerase chain reaction (RT-PCR)

An RT-PCR reaction using First-Strand cDNA Synthesis Kit (Amersham Biosciences, UK) and PCR using Takara Ex Taq™ PCR buffer (Takara Biomedicals, Japan) was performed as recommended by the manufacturers. Primers were used at final concentrations of 25 pmol/µl and used for RT-PCR reactions. The RT-PCR products were separated on 1.5% ethidium bromide agarose gel in 1xTAE buffer. The selected primers were expected to produce a 697-bp for CGMMV, and 335-bp for KGMMV fragments as the results of RT-PCR (Table 1).

Multiplex reverse transcription and polymerase chain reaction (mRT-PCR)

Two pairs of primers were combined in the mRT-PCR. In first method, 25 pmol of each of two reverse primers was mixed with each 40 ng of RNA CGMMV and KGMMV and used for RT reaction. In the second method, 25 pmol of each of two reverse primers was mixed with each 40 ng of RNA CGMMV and KGMMV and used for RT reaction. These solution were then diluted in diethyl pyrocarbonate (DEPEC)-treated water and followed by adding 1 µl DTT, 5 µl bulk first strand (First-Strand cDNA Synthesis Kit, Amersham Biosciences, UK), incubated at 37°C for 1 h. For the PCR, 25 pmol of each of the forward and reverse primers were added to 35 µl of DEPEC-treated water, 5 µl of *Taq* polymerase 10x buffer (Takara Biomedicals, Japan), 4 µl of dNTPs, 0.5 µl of *Taq* polymerase (Takara Biomedicals, Japan), and 3.5 µl of cDNA. Amplification cycles were as follows: the first denaturation for 2 min at 95°C and

Table 2. Detection of CGMMV and KGMMV by single and multiplex RT-PCR

Source plant	Sample origin	Virus	Single RT-PCR		Multiplex RT-PCR	
			CGMMV	KGMMV	CGMMV	KGMMV
Zucchini	Japan	CGMMV-W	+	-	+	-
Zucchini	Japan	KGMMV-C	-	+	-	+
Zucchini	Vietnam	Unknown	-	-	-	-
Zucchini	Indonesia	Unknown	+	+	+	+
Melon	Indonesia	KGMMV-YM	-	+	-	+
Angled loofah	Indonesia	KGMMV-YL	-	+	-	+
Cucumis metuliferus	Thailand	Unknown	-	+	-	+

then 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 52°C, and elongation for 1.5 min at 72°C. A final elongation for 5 min at 72°C was added. The amplified products were fractionated on 1.5 % agarose gel in 1xTAE buffer and ethidium bromide stained bands of interest were observed and electro-eluted using standard procedures (Sambrook *et al.*, 1989).

Results

Detection of different strains by RT-PCR

A forward primer (CG-F) and the reverse primer (CG-R) were used for the detection of CGMMV strains and the PCR products were obtained in 697 bp. These PCR products were constantly shown only in CGMMV-W and BIZ (Figure 2). This result indicates that isolate from BIZ was infected by CGMMV. On the other hand, the expected amplification bands

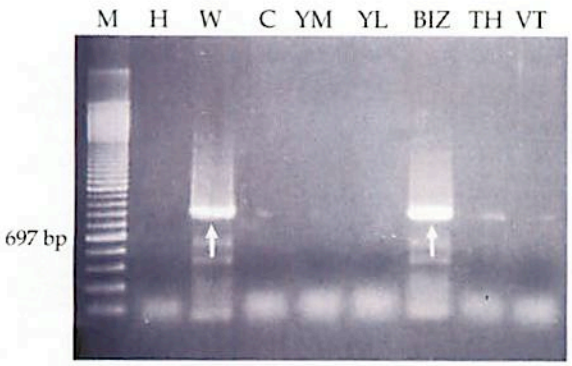

Figure 2. Gel electrophoresis of single RT-PCR products amplified by CGMMV primer from total RNA extracted from zucchini leaves showing mottle mosaic symptoms and from zucchini leaves individually infected by CGMMV and KGMMV. M: 100 bp DNA step ladder (Promega); H: healthy zucchini; W: CGMMV-W; C: KGMMV-C; YM: KGMMV- YM; YL: KGMMV-YL; BIZ: zucchini from Indonesia; TH: *C. metuliferus* from Thailand; VT: zucchini from Vietnam. A single arrow shows position of the amplified products at 697 bp.

in 697 bp were not observed for KGMMC-C, KGMMV-YM, KGMMV-YL, TH, and VT samples as well as for healthy zucchini (Figure 2), indicating they are not infected with CGMMV.

Using KG-F and KG-R primers, the PRC products were obtained in 335 bp. These products were constantly shown in KGMMV-C, KGMMV-YM, KGMMV-YL, BIZ, and TH samples (Figure 3). This result indicates that BIZ isolate was not only infected by CGMMV but also infected by KGMMV. However, expected amplification bands were not observed for VT (Figure 3). These results indicated that mottle mosaic symptoms showed on VT was not CGMMV or KGMMV.

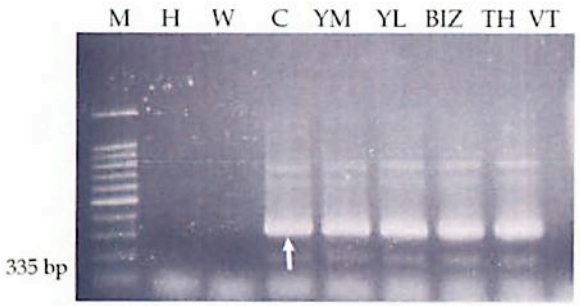

Figure 3. Gel electrophoresis of single RT-PCR products amplified by KG-F and KG-R primers from total RNA extracted from zucchini leaves showing mottle mosaic symptoms and from zucchini leaves individually infected with CGMMV and KGMMV. M: 100 bp DNA marker (Promega); H: healthy zucchini; W: CGMMV-W; C: KGMMV-C; YM: KGMMV- YM; YL: KGMMV-YL; BIZ: zucchini from Indonesia; TH: *C. metuliferus* from Thailand; VT: zucchini from Vietnam. A single arrow shows position of the amplified products at 335 bp.

Application of two RNA extraction methods

To determine whether CGMMV and KGMMV could be distinguished efficiently from each other by multiplex assay, artificial mixture of CGMMV and KGMMV samples were prepared. Using both RNA extracted from mixed leaves sample (S) and mixed RNAs (R), RT-PCR analysis was conducted with two specific primers for CGMMV (CG-F and CG-R) and KGMMV (KG-F and KG-R). As shown in Figure 4, both RNAs extracted from infected leaves and mixed RNAs could be amplified clearly by RT-PCR with CGMMV or KGMMV specific primers respectively. A coat protein gene of CGMMV showed in 697 bp (Figure 4A), whilst coat protein gene of KGMMV showed in 335 bp

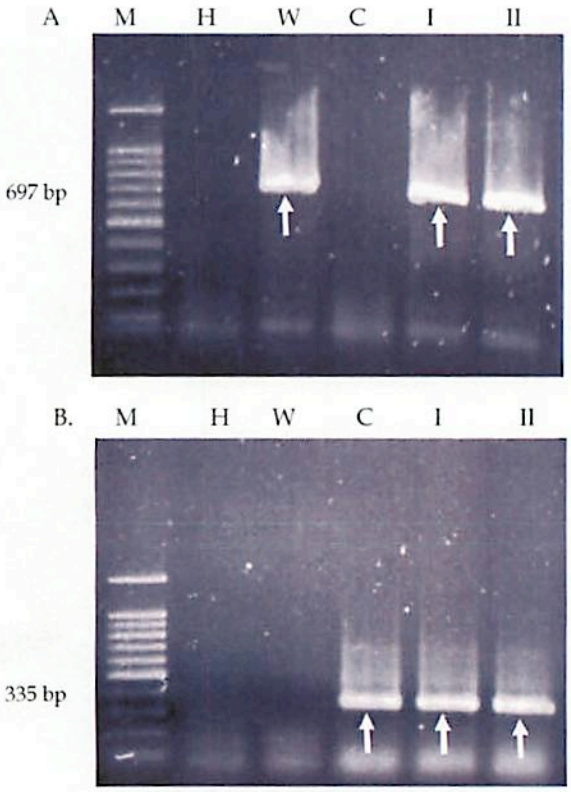

Figure 4. Gel electrophoresis of single RT-PCR detection using CGMMV and KGMMV primers for detection of CGMMV (A) and KGMMV (B). M: 100 bp DNA marker (Promega); H: healthy zucchini; W: CGMMV-W; C: KGMMV-C; YM: KGMMV-YM; I: RNA extracted from mixed infected leaves; II: Mixed RNAs. A single arrow shows CGMMV (A) and KGMMV (B).

respectively (Figure 4B). This result indicated a possibility of distinguishing either virus in mixed infected leaves and/or mixed RNA by multiplex RT-PCR.

Detection of different strains by multiplex RT-PCR

Furthermore, to conduct the application of multiplex RT-PCR for detection of artificial co-infection of CGMMV and KGMMV, two extracted RNAs were applied. The result of multiplex RT-PCR analysis revealed a single specific DNA band in 697 bp using CGMMV-W sample, while another single specific DNA band in 335 bp was shown in KGMMV-C sample. On the other hand, two specific DNA bands of CGMMV and KGMMV shown in 697 bp and 335 bp on RNA samples extracted from infected leaves (I) and RNA sample from mixed RNA (II) as presented in Figure 5A.

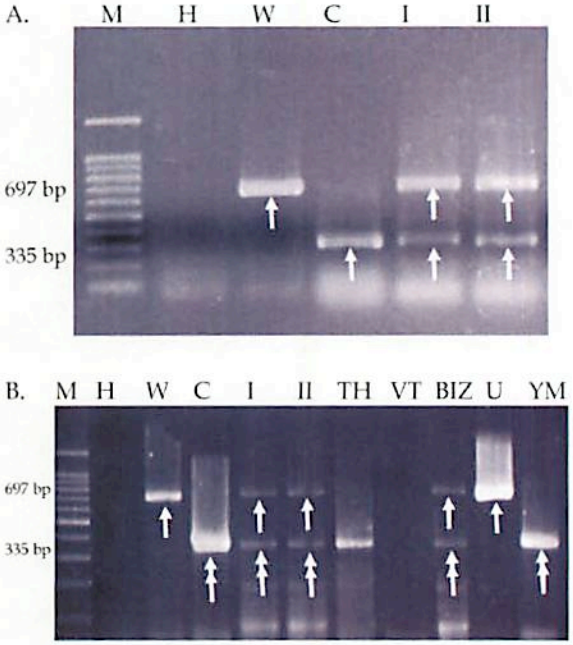

Figure 5. Gel electrophoresis of mRT-PCR products amplified by a mixture of CGMMV and KGMMV primers from total RNA extracted from zucchini leaves individually infected with CGMMV and KGMMV. A: Artificial co-infection; B: Artificial and natural co-infection; M: 100 bp DNA marker (Promega); H: healthy zucchini; W: CGMMV-W; C: KGMMV-C; I: RNAs extracted from mixed infected leaves; II: Mixed RNAs; TH: *C. metuliferus* from Thailand; VT: zucchini from Vietnam; BIZ: zucchini from Indonesia; U: CGMMV-Utsunomiya, and YM: KGMMV-YM. A single arrow shows CGMMV (A) and the double arrows show KGMMV (B).

Therefore, the results showed the possibility to apply multiplex RT-PCR for detection of natural co-infection of CGMMV and KGMMV. Using combination of CGMMV and KGMMV specific primers, multiplex RT-PCR could detect natural co-infection of CGMMV and KGMMV in BIZ sample. Two specific DNA bands for CGMMV and KGMMV were obtained by RT-PCR in BIZ sample (Figure 5B). On the other hand, a single specific DNA band for CGMMV was shown in CGMMV-W and CGMMV-U respectively, while KGMMV-C, zucchini from Thailand, and KGMMV-YM

showed a single specific DNA band for KGMMV (Figure 5B).

Discussion

Single and multiplex RT-PCR (mRT-PCR) analysis was applied for the simultaneous detection of CGMMV and KGMMV. Comparison of a single and mRT-PCR for detection of CGMMV and KGMMV in this study revealed that the two methods exhibit similar sensitivity. However, mRT-PCR is more feasible for the detection and identification of CGMMV and KGMMV in a single step reaction. The advantage of mRT-PCR is the convenience to amplify multiple sequences and identify CGMMV and/or KGMMV simultaneously. The simultaneous sensitive detection of CGMMV and KGMMV by mRT-PCR should reduce the cost and time required compared to single RT-PCR. In addition, mRT-PCR is less time consuming and has advantages of speed, suitability for large number of samples and visual examination.

Another important feature of the mRT-PCR procedure described here is that it works well with all the isolates tested in this study including two isolates of CGMMV (CGMMV-W and CGMMV-U), and four isolates of KGMMV (KGMMV-C, KGMMV-YM, KGMMV-YL, and KGMMV-TH). It is always desirable to have an internal positive control (CGMMV-W and KGMMV-C) and healthy control in both methods.

Moreover, in this study, artificial co-infection of these two viruses was prepared and carried out using two different way of viral RNAs extraction. Based on the results, it could be reported that viral RNAs for cDNA amplification by multiplex RT-PCR could be extracted from a mixture of infected leaves or separate extraction of each viruses infected leaves. This result allows the possibility of the detection of natural co-infection by multiplex RT-PCR. In the case of zucchini sample from Bali-Indonesia (BIZ), multiplex RT-PCR could detect a natural co-infection of CGMMV and KGMMV on this sample.

Results presented in this study demonstrated the application of multiplex RT-PCR to simultaneously detect CGMMV and KGMMV from cucurbit leaves using a mixture of four primers and its feasibility as a sensitive and rapid laboratory assay. Since, no multiplex RT-PCR technique has been described for the detection of CGMMV as well as KGMMV, this technique can be a good option for sensitive and reliable tool for detection of the major cucurbit infecting Tobamoviruses.

The application of multiplex RT-PCR has been reported for simultaneous detection of five seed borne viruses from legume seeds such as *Alfalfa mosaic virus* (AMV), *Bean yellow mosaic virus* (BYMV), *Clover yellow vein virus* (CYVV), CMV, and *Subterranean clover mottle virus* (SCMoV), using a mixture of nine primers and all five viruses could be detected efficiently without apparent cross-interference (Bariana *et al.*, 1994). Furthermore, multiplex RT-PCR for the simultaneous detection of different viral targets has already been proposed for three plant viruses affecting stone fruit trees such as *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), and *Apple mosaic virus* (ApMV) (Saade *et al.*, 2000). It has already been applied with success to the detection of three Polerovirus infecting sugar beet such as *Beet mild yellowing virus* (BMYV), *Brassica yellows virus* (BrYV) and *Beet chlorosis virus* (BChV) (Hauser *et al.*, 2000), three soil borne viruses infecting sugar beet such as *Beet necrotic yellow vein virus* (BNYVV), *Beet soil borne virus* (BSBV), and *Beet virus Q* (BVQ) (Meunier *et al.*, 2003), soil borne viruses of potato such as *Tobacco rattle virus* (TRV) and *Potato mop top virus* (PMTV) (Mumford *et al.*, 2000) and cereal viruses such as *Soilborne wheat mosaic virus*, and *Wheat spindle streak mosaic virus* (Gitton *et al.*, 1999). Moreover, six RNA viruses in olive trees : CMV, *Cherry leaf roll virus* (CLRV), *Strawberry latent ringspot virus* (SLRV), *Arabis mosaic virus* (AMV), *Olive latent-1 virus* (OL1V), and *Olive latent-2 virus* (OL2V) have been simultaneously detected by single-step mRT-PCR (Bertolini *et al.*, 2001).

In summary, the multiplex RT-PCR procedure developed in this study could be a

very useful method for saving time and costs during detecting CGMMV and KGMMV. The use of an assay of this kind in routine diagnosis helps to speed up and streamline the diagnostic laboratory. In addition, this assay could be also applied for preliminary virus identification combined with sequence analysis.

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