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Development of a Multiplex Polymerase Chain Reaction Assay for Detecting Five Previously Unreported Papaya Viruses for Quarantine Purposes in Korea

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There are concerns about the introduction and spread of plant pests and pathogens with globalization and climate change. As commercial control agents have not been developed for plant viruses, it is important to prevent virus spread. In this study, we developed a multiplex polymerase chain reaction (PCR) detection method to rapidly diagnose and control three DNA (papaya golden mosaic virus, Lindernia anagallis yellow vein virus, and melon chlorotic leaf curl virus) and two RNA (papaya leaf distortion mosaic virus and lettuce chlorosis virus) viruses that infect papaya. Specific primer sets were designed for the virus coat protein. Performing PCR, clear bands were observed with no non-specific reaction. Our multiplex PCR method can simultaneously detect small amounts of DNA/RNA to diagnose five viruses infecting papaya and prevent the spread of the virus.

Keywords: Lettuce chlorosis virus, Lindernia anagallis yellow vein virus, Melon chlorotic leaf curl virus, Papaya golden mosaic virus, Papaya leaf distortion mosaic virus

Papaya (family *Caricaceae*) is rich in antioxidant nutrients and B vitamins and is cultivated in tropical and subtropical regions of India, Brazil, Mexico, and Indonesia. It is cultivated on 451,181 ha worldwide, making it the third largest crop after mango and pineapple (Honoré et al., 2020; Santana et al., 2019). With climate change, the average temperature of Korea is increasing gradually, and the climate is becoming subtropical. To respond to this, the cultivation of subtropical crops has been encouraged, and the number of farms growing papaya is increasing (Jeong et al., 2020).

Various viruses have been reported to infect and damage papaya. Major viruses include papaya ringspot virus (PRSV), papaya mosaic virus, papaya leaf distortion mosaic virus (PLDMV), papaya golden mosaic virus (PaGMV), Lindernia anagallis yellow vein virus (LaYVV), melon chlorotic leaf curl virus (MCLCuV), and lettuce chlorosis virus (LCV) (Tennant et al., 2007). PRSV has caused yield losses of 70-100% in Hawaii and Mexico, while PaGMV can cause severe damage with complex infection. DNA viruses such as PaGMV, LaYVV, and MCLCuV belong to *Begomovirus*, and MCLCuV infects melons and reduces the yield. The RNA viruses PLDMV and LCV cause ongoing damage in China and the United States (Alabi et al., 2017; Bau et al., 2008; Mo et al., 2020; Zhang et al., 2017).

Plant viruses cause symptoms such as mosaic on the leaves or fruit, and the shape of the fruit can change, which lowers the value of the product and reduces the yield, causing economic loss (Wang et al., 2018). Since there are no commercial control agents for plant viral diseases, early detection is very important to avoid the spread of viral diseases (Rubio et al., 2020). The main methods of testing for plant

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viruses are serological enzyme-linked immunosorbent assays (ELISA) and molecular polymerase chain reaction (PCR). Although ELISA methods are convenient, they can fail to detect a small amount of virus (Stammler et al., 2018). Plant viruses propagate rapidly and once they are introduced and established, it is difficult to eliminate them completely. Most plant viruses are transmitted by insects, and it is very likely that the damage due to viruses will increase with expansion of insect ranges and increased winter survival rates due to climate change (Hohn, 2007; Ingwell et al., 2012; Skendžić et al., 2021).

Globalization has increased movement between countries, and trade volume is increasing steadily, including agricultural trade (Nesme et al., 2018), implying that the probability of new viruses arriving and causing damage will increase (Wu et al., 2017). Therefore, it is important to diagnose and control a very small amount of virus quickly. In this study, we developed a PCR method that can detect small amounts of RNA/DNA from three DNA viruses and two RNA viruses sensitively and rapidly by designing papaya-virus-specific primers.

In the case of three types of DNA viruses (PaGMV, LaYVV, MCLCuV) and two types of RNA viruses (PLDMC and LCV) to be used in the development of detection methods, virus clones were prepared using gene synthesis. Among the nucleic acids of five viruses registered in the National Center for Biotechnology Information (NCBI) GenBank, the isolate coat protein (CP) gene, which has a high similarity to other isolates, was selected for each virus (PaGMV-DQ318928, LaYVV-NC_009550, MCLCuV-NC_003865, PLDMV-JX974555, LCV-NC_012910). The selected gene was synthesized and inserted into the pUC57 vector to make a clone (BIONICS, Seoul, Korea) (Table 1, Fig. 1).

To produce virus-specific PCR primers, two forward and two reverse primers were prepared for the CP gene sequences of the five viruses (PaGMV, LaYVV, MCLCuV, PLDMV, and LCV) registered in NCBI GenBank (Table 1) using the primer design tool Primer3 (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) (Table 2, Fig. 2).

To develop specific primer combinations capable of detecting viruses infecting papaya, primers were selected by PCR using the virus clones as template (Fig. 3). PCR reaction mixtures were made by adding 1 μl (1 ng/μl) of virus clone mixture, 1 μl (10 pmol) of each primer mixed with DNA or RNA virus, 4 μl of EzPCR Basic 5× Master Mix (ELPIS-BIOTECH, Daejeon, Korea), and 13 μl of distilled water for a total of 20 μl. PCR of the DNA viruses was performed at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final 5 min at 72°C. For the RNA viruses, PCR was performed at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, with a final 5 min at 72°C. Clear bands were confirmed for all six primer combinations used for detecting the DNA viruses and all four primer combinations used for detecting the RNA viruses. Each PCR product was of the expected size.

Since specific reactions were confirmed in all primer combinations, multiplex PCR was performed using the first primer combination for each DNA virus. For the RNA viruses, multiplex PCR was performed using all primer combinations (Fig. 4). To determine whether each primer combination reacted with host plant genes, genomic DNA (gDNA) and RNA were extracted from healthy papaya leaves. The extracted RNA was synthesized as complementary DNA (cDNA) using a random hexamer. Performing multiplex PCR using gDNA and the PaGMV, LaYVV, and MCLCuV DNA viruses as template, the respective virus PCR products were 618, 496, and 401 bp in size, with no non-specific reactions. With multiplex PCR using cDNA and the PLDMV and LCV RNA viruses as template, the two primer combinations gave 649 and 616 bp products for PLDMV and 421 and 476 bp products for LCV, with no non-specific reaction to the host plant. The multiplex PCR products were of the expected sizes.

The diagnosis of pathogens is important for managing and preventing plant diseases. There are no treatments for plant viral diseases after infection, making it very important to diagnose the virus in advance, to reduce the damage caused by the virus and prevent the spread of disease. Electron microscopy, ELISA, molecular methods, and biosensor-based methods are used to diagnose plant viruses (Jeong et al., 2014; Mehetre et al., 2021), especially ELISA and PCR (Rinken and Kivirand, 2018). ELISA has the advantages of being able to handle large numbers of samples at a time, along with inexpensive reagents, and simple procedures, but it is expensive to produce antibodies, which are unstable if not refrigerated, and ELISA often gives false or negative results (Maciorowski et al., 2006; Sakamoto et al., 2018; Ye et al., 2016). In comparison, PCR requires expensive equipment and experts, but it can be used to analyze small samples

Table 1. Nucleic acids list of the five viruses registered in NCBI GenBank

Type	Genus	Virus	Accession no.	Site	Size (bp)
DNA	Begomovirus	PaGMV	DQ318928	Coat protein	756
	Begomovirus	LaYVV	NC_009550	Coat protein	774
	Begomovirus	MCLCuV	NC_003865	Coat protein	756
RNA	Potyvirus	PLDMV	JX974555	Coat protein	879
			MN840963	Coat protein	879
			MN840962	Coat protein	879
			MN840961	Coat protein	879
			MN840960	Coat protein	879
			MN840959	Coat protein	879
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			MN840951	Coat protein	879
			MN840950	Coat protein	879
			MN840949	Coat protein	879
			MN840948	Coat protein	879
			MN840947	Coat protein	879
			MN840946	Coat protein	879
	Crinivirus	LCV	NC_012910	Coat protein	753
			MK747245	Coat protein	753
			MN216392	Coat protein	753
			KY430286	Coat protein	843
			KX685959	Coat protein	753
			MN203150	Coat protein	753
			MN203148	Coat protein	753
			MG489895	Coat protein	753

NCBI, the National Center for Biotechnology Information; PaGMV, papaya golden mosaic virus; LaYVV, Lindernia anagallis yellow vein virus; MCLCuV, melon chlorotic leaf curl virus; PLDMV, papaya leaf distortion mosaic virus; LCV, lettuce chlorosis virus.

and has high sensitivity (Liu et al., 2019; Zhao et al., 2016). Multiple PCR is very efficient because it can detect several pathogens at once, saving time and money (Touron et al., 2005).

With climate change, it is predicted that Korea will gradually change from a temperate to a subtropical climate, and the cultivation and trade of subtropical crops are increasing accordingly (Ji et al., 2018). Papaya is the second most cultivated subtropical fruit tree in Korea after bananas.

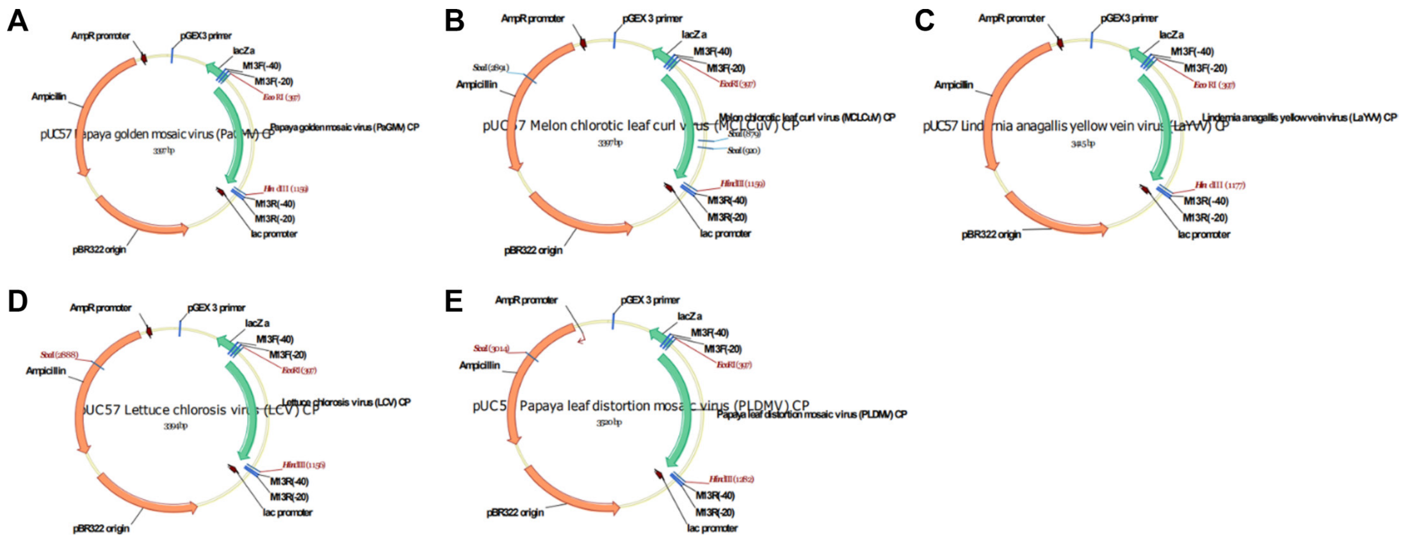

Fig. 1. Maps of the DNA/RNA virus clones. (A) pUC57-papaya golden mosaic virus CP clone, (B) pUC57-melon chlorotic leaf curl virus CP clone, (C) pUC57-Lindernia anagallis yellow vein virus CP clone, (D) pUC57-lettuce chlorosis virus CP clone, and (F) pUC57-papaya leaf distortion mosaic virus CP. CP, coat protein.

Table 2. Primer sequences designed for detecting the five viruses with multiplex PCR

Virus	Name	Sequence (5′→3′)	Size (bp)
PaGMV	PaGMV CP 87F	TGGCCCAAGAGTTAACAAGG	618
	PaGMV CP 704R	ACGGGATTAGAGGCATGTGT	
	PaGMV CP 34F	GCGGGAACCTCAAAGGTTAG	609
	PaGMV CP 642R	ATACTTCCCTGCCTCCTGGT	
MCLCuV	MCLCuV CP 93F	TAAATTCAATAAGGCCGCTGC	496
	MCLCuV CP 588R	GCGCCGGACCAGGGCTT	
	MCLCuV CP 122F	ACAGGCCCACGTACAGAAAG	332
	MCLCuV CP 453R	GAATACTTGCCCGAAATCCA	
LaYVV	LaYVV CP 49F	GTACGCCGTCGTCTGAATTT	401
	LaYVV CP 449R	GGAGTAGTGACGGGCCTTCT	
	LaYVV CP 204F	ATGTGAAGGCCCATGTAAGG	452
	LaYVV CP 655R	CAGCCTTCTCTTGGTGGTTG	
PLDMV	PLDMV CP 12F	TGCTGGCAAATyyACAGTAG	649
	PLDMV CP 660R	ACTCATGTCGGTGAGGTTCC	
	PLDMV CP 185F	TTGGATCAAGCGGATCwkTC	616
	PLDMV CP 800R	TTTCCGACTTTTCCATCCAG	
LCV	LCV CP 269F	TCAAGAAGGTTGAyGGTGAAC	421
	LCV CP 689R	CCTTTCAAAGCCTGGCACTT	
	LCV CP 264F	CGTATTCAAGAAGGTTGACGGT	479
	LCV CP 739R	CyCCTGGTGCTAGTTGAGAy	

PCR, polymerase chain reaction; PaGMV, papaya golden mosaic virus; CP, coat protein; MCLCuV, melon chlorotic leaf curl virus; LaYVV, Lindernia anagallis yellow vein virus; PLDMV, papaya leaf distortion mosaic virus; LCV, lettuce chlorosis virus.

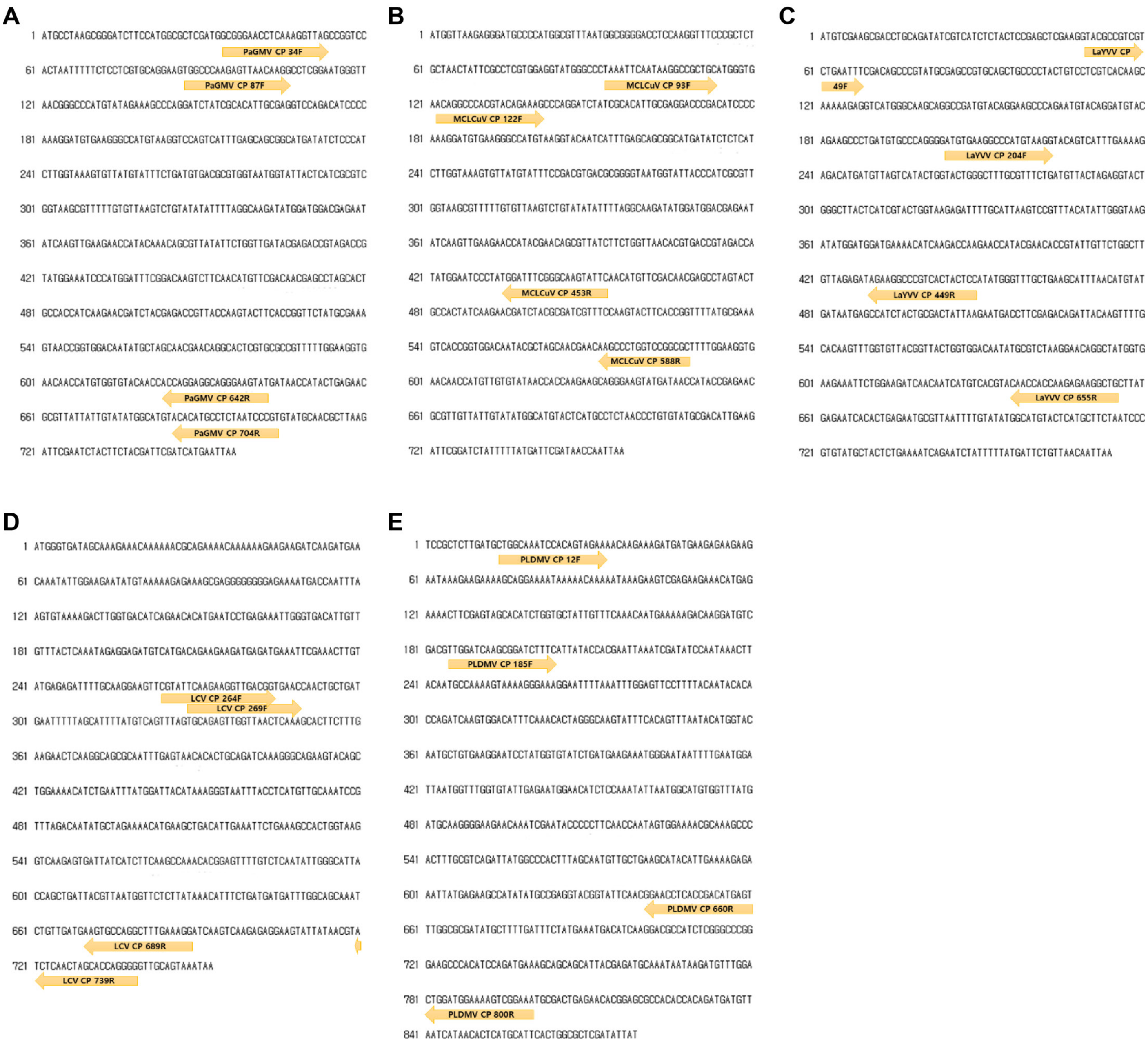

Fig. 2. Primer region of the DNA/RNA viral coat protein sequences used in this study. (A) Papaya golden mosaic virus (PaGMV), (B) melon chlorotic leaf curl virus (MCLCuV), (C) Lindernia anagallis yellow vein virus (LaYVV), (D) lettuce chlorosis virus (LCV), and (E) papaya leaf distortion mosaic virus (PLDMV). CP, coat protein.

This study examined a method for detecting three DNA (PaGMV, MCLCuV, and LaYVV) and two RNA (PLDMV and LCV) viruses that are expected to cause much damage when they infect papaya. These five viruses have not been introduced to Korea and it is difficult to acquire these viruses in Korea, so their CP genes were synthesized based on reference sequences in GenBank. The CP region of a virus is the most conserved viral gene (Diaz-Lara et al., 2020; Kutnjak et al., 2021) and is commonly used to detect viruses. Clones containing the virus CP were inserted into pUC57 vector for this study.

Virus-specific primers were designed to develop a multiplex PCR method that can simultaneously, rapidly, and accurately verify three DNA viruses and two RNA viruses that infect papaya (Table 2). All primer combinations reacted specifically with the appropriate virus when tested singly (Fig. 3). The accuracy of the selected primer sets was evaluated by performing PCR using a mixture of host plant DNA and tar-

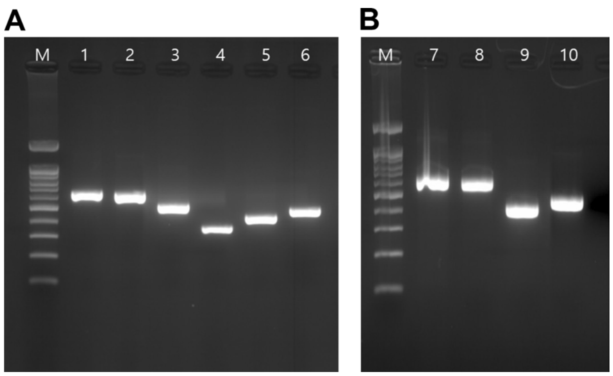

Fig. 3. Specific PCR for five viruses infecting papaya using the virus CP primer sets. (A) PCR amplification using the PaGMV, MCLCuV, and LaYVV primers with the virus clones as PCR template, (B) PCR amplification using the PLDMV and LCV primers with the virus clones as PCR template. M, size marker (100 bp DNA ladder); lane 1, PaGMV CP 87F + PaGMV CP 704R; lane 2, PaGMV CP 34F + PaGMV CP 642R; lane 3, MCLCuV CP 93F + MCLCuV CP 588R; lane 4, MCLCuV CP 122F + MCLCuV CP 453R; lane 5, LaYVV CP 49F + LaYVV CP 449R; lane 6, LaYVV CP 204F + LaYVV CP 655R; lane 7, PLDMV CP 12F + PLDMV CP 660R; lane 8, PLDMV CP 185F + PLDMV CP 800R; lane 9, LCV CP 269F + LCV CP 689R; lane 10, LCV CP 264F + LCV CP 739R. PCR, polymerase chain reaction; CP, coat protein; PaGMV, papaya golden mosaic virus; MCLCuV, melon chlorotic leaf curl virus; LaYVV, Lindernia anagallis yellow vein virus; PLDMV, papaya leaf distortion mosaic virus; LCV, lettuce chlorosis virus.

get virus as template (Fig. 4). gDNA and RNA were extracted from healthy papaya leaves, and cDNA was synthesized from the RNA. Agarose gel electrophoresis showed PCR products of the expected sizes of the three DNA (PaGMV, 618 bp; MCLCuV, 496 bp; LaYVV, 401 bp) and two RNA (PLDMV, 649 and 616 bp; LCV, 421 and 479 bp) viruses with no non-specific reaction to the host plant.

The development of transportation infrastructure and modes of transport have shortened the travel time between cities and expanded the range of movement (Biderman and Zegras, 2021; Rodrigue, 2007), which while convenient, means that harmful pathogens can also spread faster than in the past (Bradley and Altizer, 2007; Santini et al., 2018; Spence et al., 2020). Early detection of human diseases can lead to rapid treatment and prolong lifespans (Lee et al., 2004). In plant diseases, early detection leads to safe food production by preventing the spread of disease, allowing the production of healthy fruit (Dyussembayev et al., 2021). Our multiplex PCR detection method can detect 1 ng of virus and should be useful for diagnosing several viruses simultaneously and predicting and controlling viruses quickly.

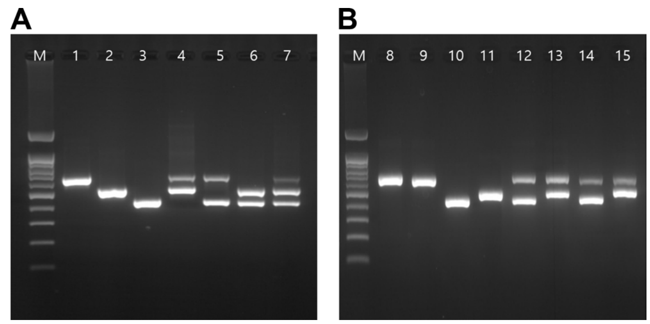

Fig. 4. Specificity test using the selected primer sets for the five viruses with multiplex PCR. (A) PCR amplification using the PaGMV, MCLCuV, and LaYVV primers with papaya gDNA and three virus clones as PCR template, (B) PCR amplification using the PLDMV and LCV primers with papaya cDNA and two viruses as PCR template. M, size marker (100 bp DNA ladder); lane 1, PaGMV CP 87F + PaGMV CP 704R; lane 2, MCLCuV CP 93F + MCLCuV CP 588R; lane 3, LaYVV CP 49F + LaYVV CP 449R; lane 4, PaGMV + MCLCuV; lane 5, PaGMV + LaYVV; lane 6, MCLCuV + LCV; lane 7, PaGMV + MCLCuV + LaYVV; lane 8, PLDMV CP 12F + PLDMV CP 660R; lane 9, PLDMV CP 185F + PLDMV CP 800R; lane 10, LCV CP 269F + LCV CP 689R; lane 11, LCV CP 264F + LCV CP 739R; lane 12, PLDMV (lane 8) + LCV (lane 10); lane 13, PLDMV (lane 8) + LCV (lane 11); lane 14, PLDMV (lane 9) + LCV (lane 10); lane 15, PLDMV (lane 9) + LCV (lane 11). PCR, polymerase chain reaction; PaGMV, papaya golden mosaic virus; MCLCuV, melon chlorotic leaf curl virus; LaYVV, Lindernia anagallis yellow vein virus; gDNA, genomic DNA; PLDMV, papaya leaf distortion mosaic virus; LCV, lettuce chlorosis virus; cDNA, complementary DNA; CP, coat protein.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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