Development of Multiplex RT-PCR for Simultaneous Detection of Garlic Viruses and the Incidence of Garlic Viral Disease in Garlic Genetic Resources

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Garlic generally becomes coinfecting with several types of viruses belonging to the *Potyvirus*, *Carlavirus*, and *Allexivirus* genera. These viruses produce characteristically similar symptoms, they cannot be easily identified by electron microscopy (EM) or immunological detection methods, and they are currently widespread around the world, thereby affecting crop yields and crop quality adversely. For the early and reliable detection of garlic viruses, virus-specific sets of primers, including species-specific and genus-specific primers were designed. To effectively detect the twelve different types of garlic viruses, primer mixtures were tested and divided into two independent sets for multiplex polymerase chain reaction (PCR). The multiplex PCR assays were able to detect specific targets up to the similar dilution series with monoplex reverse transcription (RT)-PCR. Seventy-two field samples collected by the Gyeongbuk Agricultural Technology Administration were analyzed by multiplex RT-PCR. All seventy two samples were infected with at least one virus, and the coinfection rate was 78%. We conclude that the simultaneous detection system developed in this study can effectively detect and differentiate mixed viral infections in garlic.

Keywords : garlic, multiplex RT-PCR, plant virus, simultaneous detection, virus diagnosis

Garlic (*Allium sativum* L.) is an economically important vegetable crop cultivated in Korea and in many other countries (Chung and Chang, 1979; Keusgen, 2002). Quite recently, it has gained prominence as a viable source of pharmaceutical compounds (Mikaili et al., 2013). Viral infections in garlic adversely affect the yield and bulb quality (Ahlawat, 1974; Yamashita et al., 1996). Garlic is propagated through vegetative reproduction; therefore garlic plants are readily infected by a variety of viruses, through various modes of transmission (Nagata et al., 2007). A number of reports identifying the viruses that infect the *Allium* species have been published (Koo et al., 1998; Mohamed and Young, 1981; Song et al., 1997; Sumi et al., 1993; Sward, 1991). Various filamentous viruses producing mosaic-like symptoms have been previously reported. These viruses often form complex mixtures, comprising two or more viral species, and include at least three members of the genus *Potyvirus* (*Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), and *Shallot yellow stripe virus* (SYSV)), two or more members of the genus *Carlavirus* (*Garlic common latent virus* (GCLV), and *Shallot Latent virus* (SLV)), and various members of the recently established genus *Allexivirus* (*Garlic mite-borne filamentous virus* (GMbFV), *Garlic virus A* (GarV-A), *Garlic virus B* (GarV-B), *Garlic virus C* (GarV-C), *Garlic virus D* (GarV-D), *Garlic virus E* (GarV-E), *Garlic virus X* (GarV-X), and *Shallot virus X* (ShVX)) (Brierley and Smith, 1944; Chodorska et al., 2013; Dijk, 1993; Tsuneyoshi et al., 1998a; Tsuneyoshi et al., 1998b).

Traditional phytopathological methods based on serology, host range, and symptoms are limited in their ability to

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differentiate among *Allium* viruses, owing to the presence of complex viral mixtures exhibiting similar symptoms and possessing restricted host ranges. Recent advances in molecular biology have provided effective tools for the identification and classification of viruses (Dovas et al., 2001). Several studies were undertaken previously for the classification of garlic viruses. *Garlic latent virus* (GLV) was con-

sidered identical with isolates of *Shallot latent virus* (SLV) (Dijk, 1993), *Garlic mosaic virus* (GMV) was considered identical with isolates of *Leek yellow stripe virus* (LYSV) (Yamachita et al., 1995), and *Welsh onion yellow dwarf virus* (WoYSV) was considered identical with isolates of *Shallot yellow stripe virus* (SYSV) (Chen, 2005; Van der Vlugt et al., 1998). Thus, it is necessary to effectively di-

Table 1. Analysis of 72 onion leaves collected from garlic genetic resources in Gyeongbuk Agricultural Technology Administration by multiplex RT-PCR

No.	Virus						No	Virus						
	GLV	LYSV	GCLV	OYDV	Allexi			GLV	LYSV	GCLV	OYDV	Allexi		
1	+	*	—	—	+	+	3	37	+	—	—	—	—	1
2	—	—	—	+	+		2	38	+	+	—	—	—	2
3	+	—	—	+	+		3	39	+	—	—	—	—	1
4	—	+	—	—	+		2	40	+	+	—	—	—	2
5	+	—	—	—	+		2	41	+	+	+	+	—	5
6	+	—	—	—	+		2	42	+	—	+	—	—	2
7	+	—	—	+	—		3	43	+	+	—	—	—	2
8	+	—	—	+	+		3	44	+	—	—	+	—	2
9	+	—	—	+	—		2	45	+	—	—	—	—	1
10	+	—	—	+	—		2	46	+	—	—	—	—	1
11	—	+	+	+	—		3	47	+	—	—	+	—	2
12	+	—	—	—	—		1	48	+	+	—	—	—	2
13	—	+	—	+	+		3	49	+	+	+	+	+	6
14	+	—	—	+	+		3	50	+	+	—	+	+	4
15	+	—	—	+	+		4	51	+	+	—	+	+	4
16	+	+	+	+	+		5	52	+	—	—	—	—	1
17	—	—	—	+	+		2	53	+	+	+	+	+	6
18	+	—	—	+	—		2	54	+	—	—	—	+	3
19	+	+	—	+	+		4	55	+	+	—	+	+	4
20	+	—	—	—	—		1	56	+	—	—	—	—	1
21	+	+	—	—	—		2	57	+	+	—	—	—	2
22	—	—	—	—	—		0	58	+	—	—	—	—	1
23	+	—	—	+	—		2	59	+	+	—	—	—	2
24	+	+	+	—	—		3	60	+	—	—	+	—	2
25	—	—	+	+	—		2	61	+	+	—	—	—	2
26	+	+	—	—	—		2	62	+	—	—	—	—	1
27	+	+	—	—	—		3	63	+	—	+	—	—	2
28	+	+	—	+	—		4	64	+	+	—	—	—	2
29	+	+	+	+	+		6	65	+	+	—	+	—	3
30	+	—	—	—	—		1	66	+	—	—	—	—	1
31	+	+	—	+	+		5	67	+	+	—	+	—	3
32	+	—	—	—	—		1	68	+	+	—	—	—	3
33	—	+	—	—	—		1	69	+	+	—	+	+	5
34	+	+	—	—	—		3	70	+	—	—	—	—	2
35	+	—	—	—	—		2	71	+	+	—	—	—	2
36	—	+	—	+	+		4	72	+	+	—	—	—	2

*+ positive; virus detected; — negative; virus not detected.

agnose various types of garlic viruses. Simultaneous detection assay has been demonstrated for multiplex detection of OYDV and SLV (Majumder et al., 2008), OYDV and Alllexivirus (Kumar et al., 2010) and OYDV, SLV, GarCLV and Alllexiviruses in Indian garlic accessions (Majumder et al., 2014). However, multiplex RT-PCR assays to detect effectively garlic virus that occurs in Korea have not been demonstrated. In this study, we developed two multiplex reverse transcription-polymerase chain reaction (RT-PCR) assays for the simultaneous and efficient detection of 12 economically important virus species (SLV, LYSV, OYDV, GCLV, and Alllexiviruses) except for SYSV, which is currently unavailable in Korea. The optimized multiplex RT-PCR was successfully tested by detecting these viruses in various different tissues of garlic plants.

The NCBI/GenBank database was used for identifying the most conserved regions of SLV, GCLV, LYSV, OYDV, and Alllexivirus, and species-specific primers were designed for each target virus. The sequences were aligned using DNAMAN 5.0 (Lynnon Biosoft, Quebec, Canada). Primer sets possessing similar melting temperatures, and flanking genomic regions of dissimilar sizes were selected for electrophoresis. Twenty-six types of SLV isolates, including the SLV CP gene (AB004803), and nine other members of the genus *Carlavirus*, were all used for designing the SLV species-specific primers, after consulting the NCBI/GenBank database (Supplementary Table 1). The GCLV primers were similarly designed based on the alignment of seven GCLV isolate sequences, which included the GCLV K2 CP gene (DQ520092), and eight members of the genus *Carlavirus*. The LYSV primers were designed with the help of twenty two LYSV isolate sequences and five members of the genus *Potyvirus*. The OYDV primers

were also designed from the most conserved regions of twenty two OYDV isolates and five members of the genus *Potyvirus*. Degenerate genus-specific primers for detecting alllexiviruses were designed based on the eighty one isolated sequences, which included eight members of the genus *Alllexivirus* (*Garlic mite-borne filamentous virus* (GMbFV), *Garlic virus A* (GarV-A), *Garlic virus B* (GarV-B), *Garlic virus C* (GarV-C), *Garlic virus D* (GarV-D), *Garlic virus E* (GarV-E), *Garlic virus X* (GarV-X), and *Shallot virus X* (ShVX)). Primer pairs designed for GLV, GCLV, LYSV, OYDV, and Alllexivirus were tested with monoplex RT-PCR, by using the RNA templates extracted from the leaf tissue of garlic. The amplification efficiency and specificity of these primer pairs was evaluated by visualizing the specific bands. Results showed that the amplification efficiencies of the tested primer pairs differed slightly in the detection of each virus (Figs. 1 and 2).

Total RNA was extracted from the leaves of garlic plants, using the Trizol reagent (Invitrogen; San Diego, CA, USA). RT was performed on 1 µg of total RNA extracted from leaves in a reaction volume of 10 µL, containing 1 µL of 10× First Strand Synthesis buffer (500 mM Tris-HCl pH 8.3, 30 mM MgCl₂, 750 mM KCl, 50 mM DTT), 2 µL of random primer, 2 µL of 2.5 mM dNTP, 0.5 µL of RNase Inhibitor (40 units/µL) and 0.5 µL of M-MLV reverse transcriptase (100 unit/µL) (Invitrogen, Carlsbad, CA). And 1 µL aliquot of cDNA product was added to 19 µL of PCR mixture, which consisted of 10 µL of AccuPower Multiplex PCR PreMix (Bioneer, Daejeon, Korea), 2 µL forward and reverse primers (20 µM each) and 6 µL distilled water. The RT reaction was carried out with one cycle at 94°C for 3 min, 35 cycles of PCR amplification using the step program (denaturation at 95°C for 15 s, annealing at 54°C

Table 2. List of primers used in the multiplex detection for GLV, LYSV, GCLV, OYDV and alllexivirus

Virus	Primer	Sequence (5'-3')	Product size (bp)	Target gene	Position (nt)
SLV	SL-N30	TATGGCTAACGAAGAAGAAGAACTC	203	CP*	1701
	SL-C10	CGTTCACGCTAGACAATTCAGACAT		CP	~1906
LYSV	LYS-N10	CGCATATGCAGTGATGTTTCGGTT	316	CP+3'UTR	1261
	LYS-C15	ATCAAATTCAGGCTGCTTATACAC		3'UTR	~1572
GCLV	GCL-N30	GCACCAGTGGTTTGAATGA	481	CP	666
	GCL-C40	AGCACTCCTAGAACAACCATTA		NABP	~1146
Alllexivirus	AL-N30	CAYTCHATGAAYGCBAAARATGTC	281	CP	316
	AL-C30	GGCTTATTYTGWCTAGYYTTACG		NABP	~580
OYDV	OYD-N25	CACCNATAYATAGCRGARACAGCTCT	602	Nib	500
	OYD-C06	ACTGAAATGCGCCATTATYTGCTA		CP	~1101

*CP: coat protein; UTR: untranslated region; NABP: nucleic acid binding protein; Nib: nuclear inclusion B.

for 30 s, and polymerization at 72°C for 20 s), followed by a final extension at 72°C for 10 min. A balanced amplification with similar fluorescence intensity of the bands was achieved when the primer concentrations in the multiplex RT-PCR were 0.5 μ M for GLV, 0.0625 μ M for GCLV, 0.125 μ M for LYSV, 0.25 μ M for OYDV, and 0.5 μ M for Allxivirus. The amplified products were separated by electrophoresis on a 1.5% agarose gel in 0.5 $\times$ TBE buffer (40 mM Tris-acetate and 1 mM ethylenediaminetetraacetic acid (EDTA), at pH 8.0), and stained with ethidium bromide (EtBr). The fragment sizes were determined by comparison with a 1 kb plus DNA ladder (Solgent, Daejeon, Korea).

Overall, the multiplex RT-PCR was slightly less sensitive than the monoplex RT-PCR. To overcome this weakness and to differentiate among the five viruses, the initial multiplex RT-PCR was divided into two multiplex assays. The first multiplex RT-PCR included the simultaneous detection of SLV, GCLV, and LYSV, and the second multiplex RT-PCR allowed the detection of Allxivirus and OYDV.

For designing of the first multiplex RT-PCR primer set, each of the virus-specific primer sets was selected and monoplex PCR was performed. One-step RT-PCR for the primer sets of various combinations was performed using total RNA with SLV as the template. Four of the primer sets selected exhibited high reaction intensity and hence did not show a non-specific reaction that generally interferes with the diagnosis (Fig. 1a). The primer pair GL-C30/GL-N10 showed a high amplification efficiency and specificity for SLV. Three SLV primer pairs, SL-C40/SL-N20, SL-C10/SL-N25, and SL-C10/SL-N30, showed a similar amplification efficiency and specificity for SLV (Fig. 1a). For the selection of GCLV primers, various PCR primer combinations were used and 3 GCLV-specific primer sets were selected. These sets exhibited high reaction intensity and did not show a non-specific reaction (Fig. 1b). The primer pair GCL-C40/GCL-N30 displayed a high amplification efficiency and specificity for GCLV. Two GCLV primer pairs, GCL-C30/GCL-N30 and GCL-C30-/GCL-N40, showed the specific product for GCLV (Fig. 1b). Various LYSV primer sets were also designed and two LYSV-specific primer sets were selected. Two primer sets (LYS-C15/LYS-N10 and LYS-C10/LYS-N20) amplified the expected targets for LYSV, respectively (Fig. 1c).

For designing the second multiplex RT-PCR primer set, each of the virus-specific primer sets was selected. OYDV specific primer sets were designed and three sets were selected. The amplification efficiency and specificity of these primer pairs were evaluated by visualizing the specific bands. Results showed that the amplification efficiencies of

Fig. 1. Different combination of species-specific primer for the first multiplex RT-PCR assay. Panel (A) monoplex RT-PCR for the detection of GCLV with C30-N10, C40-, C10-N25, and C10-N30 primer set. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: GCLV, Lane 2: SLV, LYSV, OYDV, and Allxivirus, Lane 3: *Pepper mottle virus* (PepMOV), Lane 4: *Tomato black ring virus* (TBRV), Lane 5: *Potato virus Y* (PVY), Lane 6: *Cowpea mild mottle virus* (CPMMV), Lane 7: *Poplar mosaic virus* (PopMV). Panel (B) monoplex RT-PCR for the detection of LYSV with C40-N30 primer set, C30-N30, and C30-N40. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: LYSV, Lane 2: SLV, GCLV, OYDV and Allxivirus, Lane 3: PepMOV, Lane 4: TBRV, Lane 5: PVY, Lane 6: *Turnip mosaic virus*, Lane 7: *Watermelon mosaic virus*. Panel (C) monoplex RT-PCR for the detection of SLV with C15-N10 and C10-N20. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: SLV, Lane 2: LYSV, GCLV, OYDV, and Allxivirus, Lane 3: PepMOV, Lane 4: TBRV, Lane 5: PVY, Lane 6: CPMMV, Lane 7: PopMV.

tested primer pairs differed slightly (Fig. 2b). The primer pair OYD-C06/OYD-N25 gave high amplification efficiency and specificity for OYDV. The two OYDV primer pairs, OYD-C06/OYD-N30 and OYD-C04/OYD-N70, showed a similar amplification efficiency and specificity for OYDV (Fig. 2b). Sequencing results confirmed that the amplified products were from the targeted viruses. An RT-PCR was developed by using a degenerate primer set specific to the CP gene, bearing the conserved region in the 81 *Allxivirus* isolates, including 8 species. A degenerate primer is a mixture of similar primers that has different bases at the variable positions. Various degenerate primer sets

Fig. 2. Different combination of species-specific primer for the second multiplex RT-PCR assay. Panel (A) monoplex RT-PCR for the detection of Alexivirus with C30-N30, C20-N30, and C20-N30 primer set. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: Alexivirus, Lane 2: *Pepper mottle virus* (PepMOV), Lane 3: *Tomato black ring virus* (TBRK), Lane 4: *Potato virus Y* (PVY). Panel (B) monoplex RT-PCR for the detection of OYDV with C06-N25, C06-N30, and C04-N70 primer set. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: OYDV, Lane 2: SLV, LYSV, and GCLV, Lane 3: LYSV and Alexivirus, Lane 4: PepMOV, Lane 5: TBRK, Lane 6: PVY, Lane 7: *Soybean mosaic virus*. Lane 8: *Turnip mosaic virus*, Lane 9: *Watermelon mosaic virus*.

for Alexivirus were designed and three degenerate primer sets (Al-C30/Al-N30, Al-C20/Al-N30, and Al C20/Al-N30) amplified the expected targets for Alexivirus. Finally, the primer pairs SL-C10/SL-N30 for SLV, LYS-C15/LYS-N10 for LYSV, and GCL-C40/GCL-N30 for GCLV were selected for the first multiplex RT-PCR set (Fig. 2a). AL-C30/AL-N30 for Alexivirus and OYD-C06/OYD-N25 for OYDV were selected for the second multiplex RT-PCR set (Fig. 2a). These primer pairs generated PCR products of different sizes, which could be easily differentiated by agarose gel electrophoresis.

To compare the relative sensitivity of monoplex RT-PCR assays and the multiplex RT-PCR assay, a series of 10-fold dilutions of cDNAs generated from the garlic-extracted total RNA were subjected to RT-PCR with the designed species-specific primer sets, either separately or together. The detection limits of the monoplex RT-PCR assays were 10^{-4} for GCLV, LYSV, and OYDV, and a slightly lower limit of 10^{-3} was observed for SLV and Alexivirus. In the multiplex RT-PCR assay, most of viruses were detected after the template cDNA of the 5 viruses was diluted 1000-

Fig. 3. Two independent multiplex RT-PCR assays for the detection of SLV, GCLV, LYSV, OYDV and Alexivirus. The first multiplex RT-PCR included the simultaneous detection of SLV, GCLV, and LYSV, and the second multiplex RT-PCR allowed the detection of Alexivirus and OYDV. M: 1 kb plus ladder (Solgent, Daejeon, Korea), Lane 1: SLV, Lane 2: LYSV, Lane 3: GCLV, Lane 4: SLV and LYSV, Lane 5: SLV and GCLV, Lane 6: LYSV and GCLV, Lane 7: SLV, LYSV and GCLV, Lane 8: Alexivirus, Lane 9: OYDV, Lane 10: Alexivirus and OYDV.

fold; however, the detection limits for SLV and Alexivirus was slightly lower. This reduction was not significant for detecting SLV and Alexivirus in the multiplex RT-PCR. In all cases, the monoplex and multiplex RT-PCRs were able to detect specific targets up to a similar dilution. These results indicated that the multiplex RT-PCR assay for the 5 viruses was suitable for the simultaneous detection of these viruses.

Fig. 4. Comparison of the sensitivity of the monoplex and multiplex RT-PCR assay. RT-PCR assays were performed using SLV, LYSV and GCLV-specific primer sets (A) and Alexivirus and OYDV-specific primer set (B). RT-PCR Fragments 481 bp, 312 bp, 206 bp, 602 bp and 265 bp of GCLV, LYSV, SLV, OYDV and Alexivirus were amplified from 10-fold serial dilutions of the cDNAs generated from total RNA extracted from garlic. M: 1 kb plus ladder (Solgent, Daejeon, Korea).

To validate the multiplex RT-PCR assay, 72 garlic samples were collected from a demonstration field of garlic germplasm, collected from different provinces by the Gyeongbuk Agricultural Technology Administration, and analyzed by multiplex RT-PCR for the identification of garlic-infecting viruses. All 72 samples were infected by at least one of the viruses and the coinfection rate reached 78%. The coinfection rates were as follows: 16 samples (22.2%) were coinfecting by a single virus, 32 samples (44.4%) were coinfecting by two viruses, 12 samples (16.7%) were coinfecting by three viruses, 8 samples (11.1%) were coinfecting by four viruses, and 4 samples (6.9%) were coinfecting by five viruses, respectively. Thus, a number of garlic plants were infected by two or more viruses, on an average. SLV was detected in 64 (89%), LYSV in 36 (50%), OYDV in 34 (47%), *Allexivirus* in 24 (33%), and GCLV in 10 (14%) samples.

Most of the garlic plants contained a complex mixture of viruses. For the rapid, simple, and simultaneous detection of garlic viruses, it is necessary to develop more effective diagnostic systems. In a previous study, a duplex RT-PCR was developed for the simultaneous detection of OYDV and SLV (Majumder et al., 2008), OYDV and *Allexivirus* (Kumar et al., 2010) and LYSV and OYDV (Taskin et al., 2013). However, it was not sufficient for the detection of most of the garlic viruses. The two highly effective multiplex RT-PCR assays reported in this study can be used for the rapid and accurate identification of garlic-associated viruses and are especially useful for analyzing coinfections in garlic plants.

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