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Development of an RT-PCR assay and its positive clone for plant quarantine inspection of *American plum line pattern virus* in Korea

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Abstract

American plum line pattern virus (APLPV), a member of the genus *Ilarvirus* in the family *Bromoviridae*, is one of the plant quarantine pathogens in Korea. In this study, 15 candidate primer sets were designed and examined to develop a reverse transcription polymerase chain reaction (RT-PCR) assay for plant quarantine inspection of APLPV. Using APLPV-infected and healthy samples, the primer sets were assessed for APLPV detection. To confirm the occurrence of nonspecific reactions, six ilarviruses (*Apple mosaic virus*, *Asparagus virus 2*, *Blueberry shock virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, and *Tobacco streak virus*) and 10 target plants (*Prunus mume*, *P. yedoensis*, *P. persica*, *P. armeniaca*, *P. dulcis*, *P. tomentosa*, *P. avium*, *P. glandulosa*, *P. salicina*, and *P. cerasifera*) were examined. Finally, two primer sets were selected. These primer sets could generate the expected amplicons even with at least 1 ng of the total RNA template in concentration-dependent amplifications. In addition, a positive clone was developed for use as a positive control in the abovementioned RT-PCR assay.

Key words: APLPV (*American plum line pattern virus*), diagnosis, plant quarantine, plant virus, RT-PCR (reverse transcription polymerase chain reaction)

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Introduction

American plum line pattern virus (APLPV) belonging to the genus *Ilarvirus* in the family *Bromoviridae* has a tripartite genome (RNA1, RNA2, and RNA3) (Herranz et al., 2008; Pallas et al., 2013). RNA1 and RNA2 encode proteins associated with viral replication, and RNA3 encodes a movement protein (MP) and a coat protein (CP) (Herranz et al., 2008; Pallas et al., 2013). APLPV was first reported in North America and has been recently recorded in European and Mediterranean regions (Herranz et al., 2008; Candresse et al., 2017). The virus was detected in *Prunus mume*, *P. persica*, and *P. serrulate*, showing chlorotic lines on the leaves (EPPO, 2006). Some cultivars, however, were asymptomatic (Alayasa et al., 2003). APLPV has been listed as an A1 plant pathogen by the European and Mediterranean Plant Protection Organization (EPPO) (Rwahnih et al., 2004). In

addition, APLPV has also been designated as a plant quarantine virus in Korea, and some *Prunus* spp. (plum, peach, apricot, and almond) imported from foreign countries are examined for the presence of this virus (Animal and Plant Quarantine Agency, www.qia.go.kr).

Various methods are used to confirm plant virus infections, such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), DNA chip, and high-throughput sequencing technology (Boonham et al., 2014; Jeong et al., 2014). ELISA has been the primary method to detect the presence of plant viruses in the past when molecular biology techniques were not common. Currently, PCR assays are mainly used in various fields, including diagnostic fields, because they require shorter test duration and have better sensitivity and specificity than ELISA (Lee et al., 2013a; 2013b; Shin et al., 2017). Furthermore, because the PCR amplicons can be further analyzed through sequencing technology, more accurate experimental results can be obtained (Lee et al., 2021). Previous studies have attempted the development of various detection methods, including a PCR assay, to detect APLPV (Scott and Zimmerman, 2001; Alayasa et al., 2003; Sanchez-Navarro et al., 2005). However, despite their usability in virus detection, these methods were not developed for the purpose of plant quarantine inspections, as described by Lee et al. (2021). Therefore, in this study, we developed a one-step RT-PCR assay for the detection of APLPV.

Materials and Methods

Collection of samples

To collect virus-positive samples and target plants, we purchased positive and negative controls for ELISA (Agdia, Elkhart, IN, USA) and healthy seeds and seedlings. The plant virus-positive samples were as follows: APLPV, *Apple mosaic virus* (ApMV), *Asparagus virus 2* (AV2), *Blueberry shock virus* (BlShV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), and *Tobacco streak virus* (TSV). The plant samples were as follows: *Prunus mume*, *P. yedoensis*, *P. persica*, *P. armeniaca*, *P. dulcis*, *P. tomentosa*, *P. avium*, *P. glandulosa*, *P. salicina*, and *P. cerasifera*.

Design of candidate primers

To design candidate primers for the detection of APLPV, all 33 nucleotide sequences (4 in RNA1, 6 in RNA2, and 23 in RNA3) of APLPV registered in the National Center for Biotechnology Information (NCBI) GenBank were collected and aligned using CLC Main Workbench 6 (QIAGEN, Germantown, MD, USA). Conserved regions (candidate sequences) to be designed as primer sequences were searched through analysis of alignments of all the APLPV nucleotide sequences. The candidate sequences were analyzed to verify the binding specificity for APLPV through NCBI BLASTn analysis (https://blast.ncbi.nlm.nih.gov).

Total RNA extraction and RT-PCR condition

Total RNA was extracted from the samples using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany). One-step RT-PCR assay was performed using the total RNA, random N25 primer (50 pmol), each primer set (10 pmol), and AccuPower® RT-PCR PreMix (Bioneer, Daejeon, Korea). At every step for verification, we used three RT-PCR premixture

products manufactured by different companies, but only the results derived from using the AccuPower® RT-PCR PreMix were presented as data. The one-step RT-PCR began with cDNA synthesis at 42℃ for 1 h, followed by RT enzyme inactivation at 95℃ for 15 min. PCR was performed for 32 cycles as follows: 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min, followed by an additional extension at 72℃ for 5 min.

The four verification stages for selection of optimal primer sets

In this study, we developed a one-step RT-PCR assay for the detection of APLPV. The expected primer characteristics were as follows: (i) the primers used should be able to accurately detect almost all existing isolates with genetically divergent sequences; (ii) the primers should not bind nonspecifically with the genome of other closely related viruses or (iii) imported plants to be tested; and (iv) finally, the selected primers should have a high detection ability even at a low titer of the target virus.

Development of a positive clone

The sequences of each pair of primers were arranged according to their expected amplicon sizes. The positive clone comprised sequences of primers for detection of six plant viruses including APLPV and artificial sequences unrelated to plant viruses. An artificial DNA fragment was synthesized on the basis of DNA synthesis technology and cloned into the pUCIDT-AMP vector by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Results and Discussion

Pairwise comparisons between APLPV isolates and combinations of candidate primers

Viruses have sequence variation among isolates within each species (Lim et al., 2015; 2019; Oh et al., 2019), so this point should be taken into account when developing diagnostic methods. Prior to primer design, pairwise comparisons of the sequences of MP and CP genes of RNA3 were performed to analyze the genetic variation across APLPV isolates. The pairwise comparisons showed > 97% nucleotide sequence homologies in the MP and CP nucleotide sequences (Fig. 1). We searched conserved regions based on alignments of the nucleotide sequences of APLPV isolates, and seven conserved regions in RNA2 and eight in RNA3 were searched for primer design. Since RNA1 has a relatively small number of registered nucleotide sequences, its regions were not searched. The BLASTn searches of the 15 candidate sequences revealed the binding specificity of candidate sequence no. 6 for *Ageratum latent virus* (another member of the genus *Ilarvirus*) as well as APLPV (Supplementary Table 1). The candidate sequences were matched to generate amplicons of expected size (300 - 900 bp), but candidate sequence no. 7 was not available. Consequently, a total of 15 candidate primer sets were developed (Supplementary Table 2 and Supplementary Fig. 1).

(A)

		1	2	3	4	5	6	7	8	9	10	11
AF235166	1											
LC496471	2	97.71										
KY883317	3	98.74	98.06									
EF494421	4	99.08	98.17	98.97								
EF494418	5	98.85	97.94	98.97	99.31							
EF494415	6	98.74	98.17	98.85	99.20	98.97						
EF494419	7	98.85	97.71	98.51	99.08	99.08	98.74					
EF494420	8	98.63	97.71	98.51	98.85	98.63	98.51	98.40				
EF494417	9	98.28	97.37	98.17	98.74	98.74	98.40	98.97	98.28			
EF494414	10	98.28	97.37	98.17	98.74	98.74	98.40	98.97	98.28	99.77		
EF494416	11	98.05	97.14	97.94	98.05	97.94	97.94	98.17	97.82	97.37	97.59	

(B)

		1	2	3	4	5	6	7	8	9	10	11
AF235166	1											
LC496471	2	98.32										
KY883317	3	98.62	97.86									
EF494412	4	98.32	98.47	98.17								
EF503724	5	98.47	98.62	98.62	99.24							
EF494413	6	98.78	98.93	98.32	99.24	99.08						
EF494407	7	98.62	98.62	98.17	98.93	98.78	99.39					
EF494411	8	98.62	98.78	98.17	98.78	98.93	99.24	98.93				
EF494410	9	98.17	98.32	97.71	98.93	98.78	98.78	98.47	98.62			
EF494408	10	98.01	98.47	97.55	98.78	98.62	98.62	98.32	98.47	99.85		
EF494409	11	97.71	98.17	97.40	98.47	98.32	98.62	98.32	98.17	98.01	98.17	

Fig. 1. Pairwise comparisons using complete sequences of MP and CP genes of APLPV isolates. (A) A pairwise comparison based on an alignment of 11 complete MP-coding sequences, and (B) a pairwise comparison based on an alignment of 11 complete CP-coding sequences. The blue and red colors represent the comparison gradient, with relatively low homology in blue and high homology in red. MP, movement protein; CP, coat protein; APLPV, *American plum line pattern virus*.

The first verification stage

First, the specificity of the candidate primer sets was verified using positive and negative controls for APLPV. In this verification stage, 13 candidate primer sets (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15) were selected (Supplementary Fig. 2). Primer sets 1 and 2, which produced a weak target band and nonspecific reaction in the other RT-PCR premixture tests (data not shown), were excluded.

The second verification stage

Afterward, we confirmed whether the candidate primer sets could nonspecifically bind to the genomes of other closely related viruses. In this verification stage, six ilarviruses (ApMV, AV2, BlShV, PDV, PNRSV, and TSV) were used. The results of the one-step RT-PCR revealed that the use of five candidate primer sets (3, 4, 5, 6, and 12) resulted in nonspecific smear bands (Fig. 2). Consequently, eight candidate primer sets (7, 8, 9, 10, 11, 13, 14, and 15) were selected for the subsequent stages of verification.

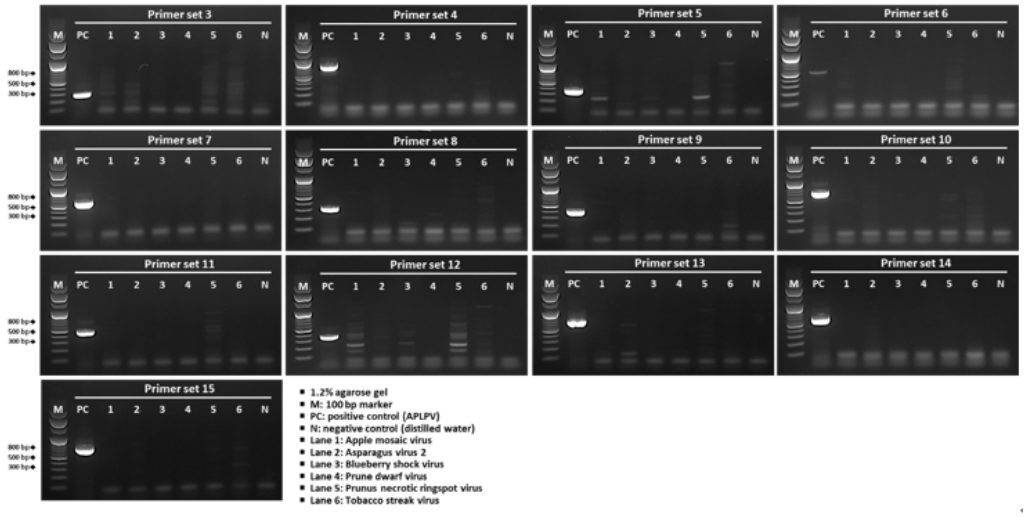

Fig. 2. Polymerase chain reaction results for nonspecific reactions in six ilarviruses. Six closely related viruses belonging to the genus *Ilarvirus* (*Apple mosaic virus*, *Asparagus virus 2*, *Blueberry shock virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, and *Tobacco streak virus*) were examined to confirm the occurrence of nonspecific reactions.

The third verification stage

Some *Prunus* species (*P. mume*, *P. yedoensis*, *P. persica*, *P. armeniaca*, *P. dulcis*, *P. tomentosa*, *P. avium*, *P. glandulosa*, *P. salicina*, and *P. cerasifera*) imported into Korea are tested for APLPV infections. During plant quarantine inspections, nonspecific products derived from the host plants may interfere with the test results. Therefore, we confirmed whether nonspecific amplicons were generated in the RT-PCRs using the target plant samples. Primer sets 8 and 13 were excluded because of nonspecific band formation (Fig. 3). In addition, four primer sets (10, 11, 14, and 15) were excluded because of observed inconsistent reactivity such as weak reactivity from the APLPV-infected sample (Fig. 3). Thus, two candidate primer sets (7 and 9) were selected.

The fourth verification stage

Finally, concentration-dependent amplification tests were performed using primer sets 7 and 9. The initial amount of total RNA extracted from the APLPV-infected sample was 100 ng, and six additional templates were prepared through 1/10 serial dilutions. Primer sets 7 and 9 generated amplicons up to 1 and 0.1 ng of the total RNA template, respectively (Supplementary Fig. 3). In this study, we used the positive samples for ELISA because of the difficulty associated with collecting and handling plants infected with APLPV, a virus designated and regulated as a plant quarantine pathogen in Korea. Considering that the positive control was a freeze-dried sample, sensitivity in fresh samples is expected to be much higher.

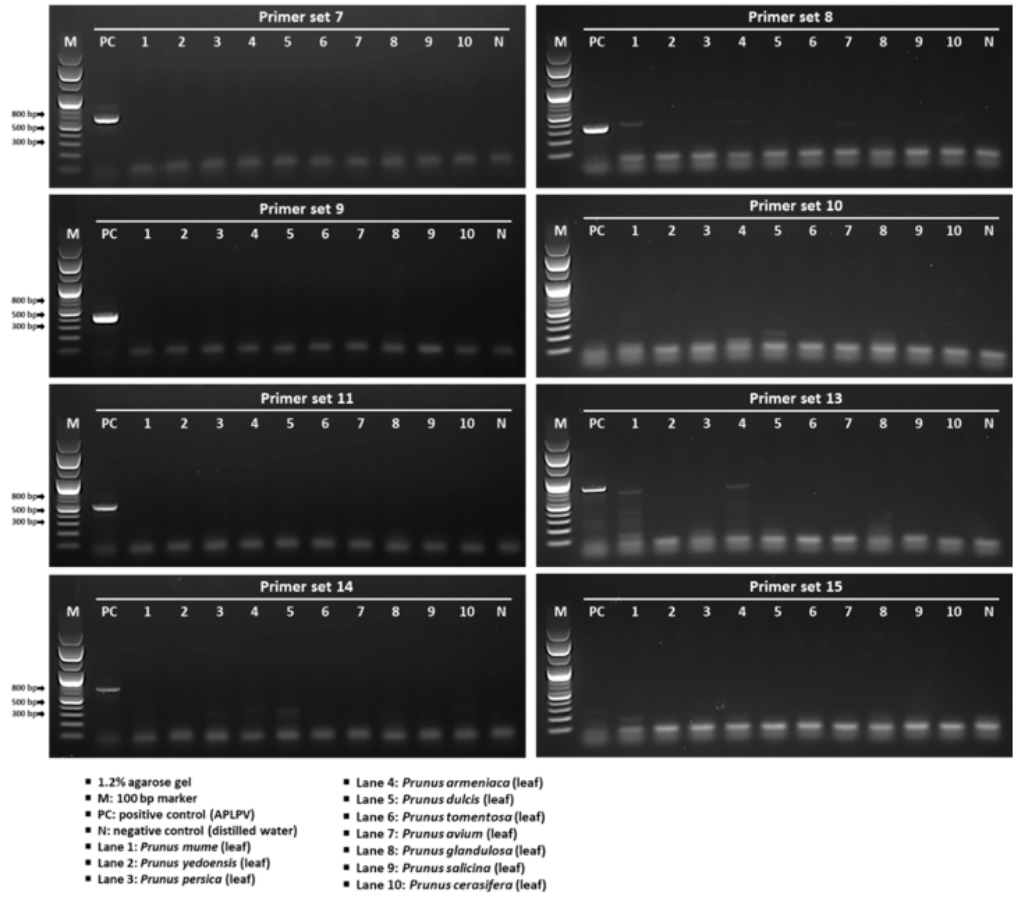

Fig. 3. Polymerase chain reaction results for nonspecific reactions in 10 target plants. In total, 10 *Prunus* spp. (*P. mume*, *P. yedoensis*, *P. persica*, *P. armeniaca*, *P. dulcis*, *P. tomentosa*, *P. avium*, *P. glandulosa*, *P. salicina*, and *P. cerasifera*), which are target imported plants for *American plum line pattern virus* (APLPV) inspections in Korean plant quarantine, were examined to confirm the occurrence of nonspecific reactions.

Verification for efficiency of positive clone

The use of positive and negative controls in PCR assays can improve the reliability of the inspection process and results. However, it is challenging to obtain plant quarantine pathogens through collection and purchase. Moreover, laboratory contamination due to the frequent use of nucleic acids extracted from positive samples makes it difficult to analyze the test results. To solve this problem, we developed a plasmid using DNA synthesis technology for use as a positive control in PCR (Fig. 4a). To verify the use of the plasmid as a positive control in PCR, PCR was performed with 1 and 0.1 ng of the positive clone. The two primer sets (no. 7 and 9) generated PCR products of the expected sizes without any nonspecific bands (Fig. 4b). Thus, the positive clone could be used for the two APLPV-specific primer sets and could additionally be applied to primer sets for detecting five other viruses (data not shown). This result indicated that the positive clone developed on the basis of DNA synthesis technology can be used efficiently as a positive PCR control in PCR assay including plant quarantine inspections.

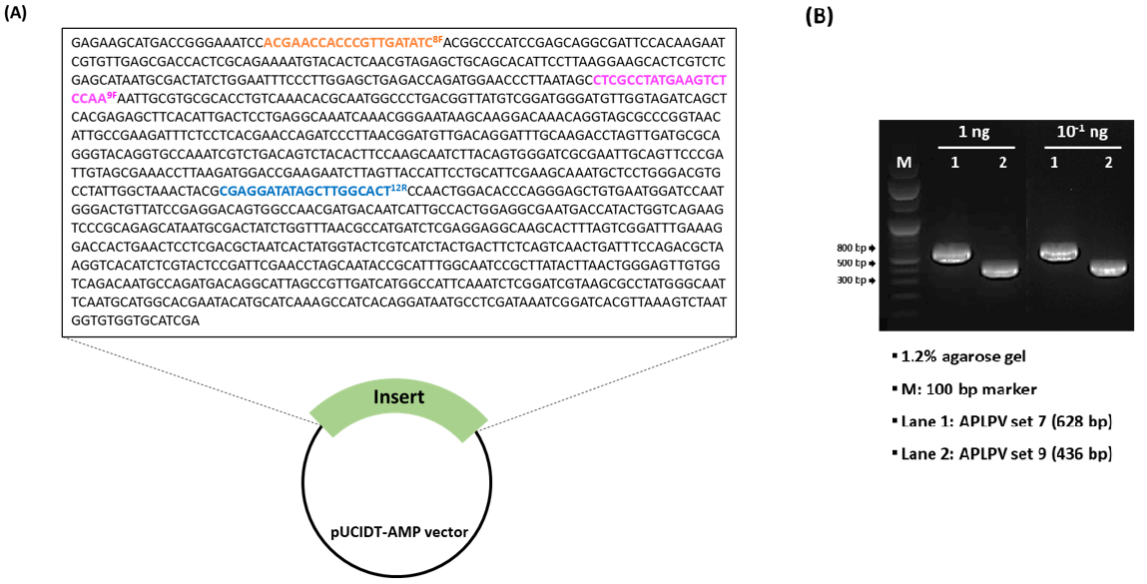

Fig. 4. Development of a positive clone for use as a positive control in PCR for APLPV detection. (A) The positive clone was synthesized by arranging primer sequences that correspond with the expected PCR amplicon sizes. (B) Concentration-dependent amplification was performed using 1 and 0.1 ng of the positive clone. PCR, polymerase chain reaction; APLPV, *American plum line pattern virus*.

Conclusion

In this study, we selected two primer sets to detect APLPV and developed a positive clone for use as the control template in an RT-PCR assay. The two primer sets and the positive clone can be efficiently used to detect the APLPV infection of target imported plants in Korean plant quarantine inspections. Furthermore, the primer sets designed and examined in this study will be useful for APLPV detection in various other fields.

Conflict of Interests

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

Ethical approval

This article does not describe any studies with human participants or animals performed by any of the authors.

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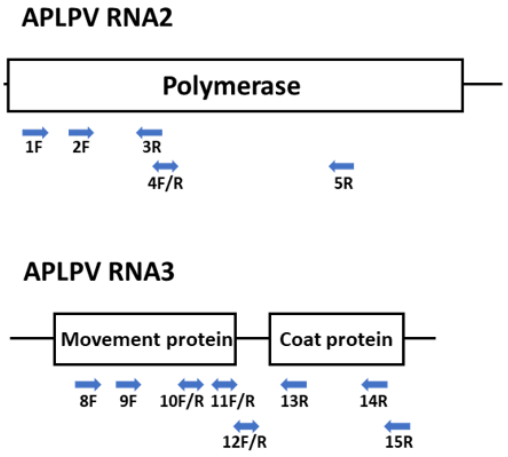
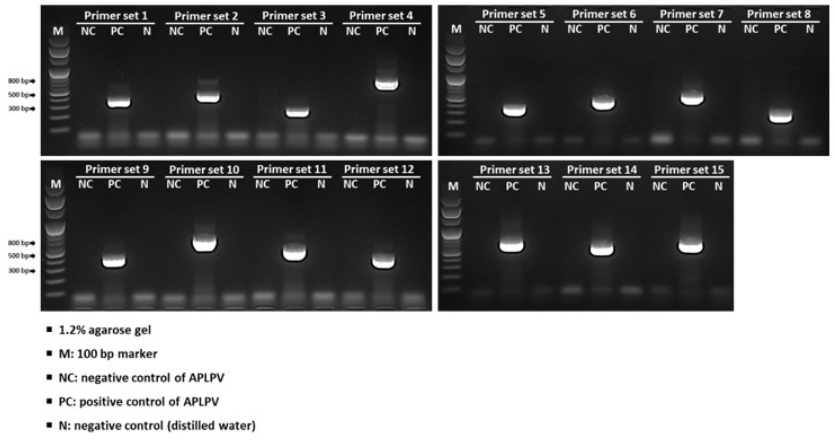
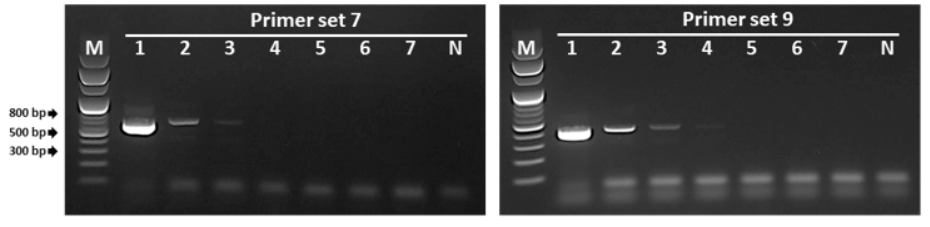
Supplementary Data



Supplementary Fig. 1. Locations of candidate primers designed and verified in this study. Position of candidate primers on maps of the segmented *American plum line pattern virus* (APLPV) genomic RNAs. F, forward primer; R, reverse primer.



Supplementary Fig. 2. Verification of candidate primers designed for *American plum line pattern virus* (APLPV) detection. In total, 15 candidate primer sets were examined by one-step reverse transcription polymerase chain reaction (RT-PCR) assay using APLPV-infected and healthy samples.



Lane	1	2	3	4	5	6	7	N
Concentration (ng)	100	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0

Supplementary Fig. 3. Concentration-dependent amplification of the final primer sets. The initial amount of the total RNA template was 100 ng, and six additional templates were prepared through 1/10 serial dilutions (N, negative control).

Supplementary Table 1. Results of the NCBI BLASTn search using 15 candidate sequences for *American plum line pattern virus* (APLPV) detection.

No.	Query sequence (15 candidate regions)	Locus[z]	Results of the NCBI BLASTn searches			
			Subject description	The number of subject	Identity (%)	Coverage (%)
RNA2						
1	GGT TCA AGA GTA TTA CCG TC	241-260	APLPV	4	100	100
2	GAA GTT GAG GAC GGA GGT CA	450-469	APLPV	4	100	100
3	CAA TAC TGT CGT GAA GCA GG	644-663	APLPV	4	100	100
4	CGT GTC TGA TGC CAT CGA AG	731-750	APLPV	4	100	100
5	AAG ATC TAC GAC AGG GAT GC	1569-1588	APLPV	5	100	100
6	TGA TGC GTG CAC TTA CTT GG	1628-1647	APLPV	5	100	100
			Ageratum latent virus	1	100	90
7	CCT CGC GAT AAG GAA CAG TT	1767-1786	APLPV	5	100	100
RNA3						
8	ACG AAC CAC CCG TTG ATA TC	458-477	APLPV	11	100	100
9	CTC GCC TAT GAA GTC TCC AA	650-669	APLPV	11	100	100
10	GTT TGT CAA TGC CAC GAT GG	832-851	APLPV	11	100	100
11	GAC CGA TTA TCG TTC CGT CA	966-985	APLPV	11	100	100
12	CGA GGA TAT AGC TTG GCA CT	1066-1085	APLPV	14	100	100
13	CTC AAC GTA GAG CTG CAG CA	1330-1349	APLPV	14	100	100
14	TTC AGA GAG GGT AGG CGA CT	1704-1723	APLPV	12	100	100
15	GCA TTC GAA GCA AAT GCT CC	1806-1825	APLPV	12	100	100

[z] Locations of sequences are based on the reference genomic sequences of APLPV (GenBank acc. nos. NC_003452 [RNA2] and NC_003453 [RNA3]).

Supplementary Table 2. Combinations of candidate primers designed for the RT-PCR-based detection of *American plum line pattern virus* (APLPV).

Set no.	Primer name	Sequence (5' to 3')	Locus[z]	Expected size (bp)
RNA2				
1	APLPV_1F	GGT TCA AGA GTA TTA CCG TC	241-260	423
	APLPV_3R	CCT GCT TCA CGA CAG TAT TG	663-644	
2	APLPV_1F	GGT TCA AGA GTA TTA CCG TC	241-260	510
	APLPV_4R	CTT CGA TGG CAT CAG ACA CG	750-731	
3	APLPV_2F	GAA GTT GAG GAC GGA GGT CA	450-469	301
	APLPV_4R	CTT CGA TGG CAT CAG ACA CG	750-731	
4	APLPV_4F	CGT GTC TGA TGC CAT CGA AG	731-750	858
	APLPV_5R	GCA TCC CTG TCG TAG ATC TT	1588-1569	
RNA3				
5	APLPV_8F	ACG AAC CAC CCG TTG ATA TC	458-477	394
	APLPV_10R	CCA TCG TGG CAT TGA CAA AC	851-832	
6	APLPV_8F	ACG AAC CAC CCG TTG ATA TC	458-477	528
	APLPV_11R	TGA CGG AAC GAT AAT CGG TC	985-966	
7	APLPV_8F	ACG AAC CAC CCG TTG ATA TC	458-477	628
	APLPV_12R	AGT GCC AAG CTA TAT CCT CG	1085-1066	
8	APLPV_9F	CTC GCC TAT GAA GTC TCC AA	650-669	336
	APLPV_11R	TGA CGG AAC GAT AAT CGG TC	985-966	
9	APLPV_9F	CTC GCC TAT GAA GTC TCC AA	650-669	436
	APLPV_12R	AGT GCC AAG CTA TAT CCT CG	1085-1066	
10	APLPV_9F	CTC GCC TAT GAA GTC TCC AA	650-669	700
	APLPV_13R	TGC TGC AGC TCT ACG TTG AG	1349-1330	
11	APLPV_10F	GTT TGT CAA TGC CAC GAT GG	832-851	518
	APLPV_13R	TGC TGC AGC TCT ACG TTG AG	1349-1330	
12	APLPV_11F	GAC CGA TTA TCG TTC CGT CA	966-985	384
	APLPV_13R	TGC TGC AGC TCT ACG TTG AG	1349-1330	
13	APLPV_11F	GAC CGA TTA TCG TTC CGT CA	966-985	758
	APLPV_14R	AGT CGC CTA CCC TCT CTG AA	1723-1704	
14	APLPV_12F	CGA GGA TAT AGC TTG GCA CT	1066-1085	658
	APLPV_14R	AGT CGC CTA CCC TCT CTG AA	1723-1704	
15	APLPV_12F	CGA GGA TAT AGC TTG GCA CT	1066-1085	760
	APLPV_15R	GGA GCA TTT GCT TCG AAT GC	1825-1806	

[z] Locations of primers are based on the reference genomic sequences of APLPV (GenBank acc. nos. NC_003452 [RNA2] and NC_003453 [RNA3]).