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Incidence of virus diseases and RT-PCR detection of *Daphne*-infecting viruses in Korea

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Abstract Reverse transcription-polymerase chain reaction (RT-PCR) was developed for the detection of viruses from *Daphne odora* plants in this study. Five sets of primers were designed for the specific detection of five different viruses, *Daphne virus Y* (DVY, Potyvirus), *Cucumber mosaic virus* (CMV, Cucumovirus), *Daphne virus S* (DVS, Carlavirus), *Cycas necrotic stunt virus* (CNSV, Nepovirus), and *Watermelon mosaic virus* (WMV, Potyvirus) in collected *Daphne* plants. RT-PCR could successfully detect the viruses from the *Daphne* plants. Two potyviruses (DVY and WMV), CNSV, CMV and DVS were frequently detected in >85% samples examined in this study. CNSV was the most prevalent virus (78%), and the infection rate for the three viruses WMV, CMV and DVS was >60%. These results demonstrate that virus diseases on *Daphne* plants are extremely severe and widespread in Korea. It is especially notable that mixed virus infections with more than three different viruses were very common and 12% of *Daphne* plants were infected by the five viruses. The results suggest that five primer sets developed in this study can successfully detect the target viruses and can be useful for the monitoring of virus incidence and indexing of the virus-free *Daphne* plants.

Keywords *Cucumovirus* · *Carlavirus* · *Daphne odora* · Detection · Incidence · Mixed infection · *Nepovirus* · *Potyvirus* · RT-PCR · Virus indexing

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Introduction

Daphne is a one of the popular evergreen ornamental shrubs worldwide. The genus *Daphne* belongs to the family *Thymelaeaceae*. Producers commercially propagate many varieties of *Daphne* plants by cuttings in Korea; the risk to *Daphne* plants being infected with several viruses is therefore high when sources of plants are infected.

In particular, *Daphne*-infecting viruses such as *Cycas necrotic stunt virus* (CNSV, *Nepovirus*), *Carnation mottle virus* (CaMoV, *Carmovirus*), *Cucumber mosaic virus* (CMV, *Cucumovirus*), *Daphne virus S* (DVS, *Carlavirus*), *Daphne virus X* (DVX, *Potexvirus*), *Daphne virus Y* (DVY, *Potyvirus*), *Daphne mosaic virus* (DapMV, *Potyvirus*), a tobamovirus, *Alfalfa mosaic virus*, *Arabis mosaic virus*, *Tobacco ringspot virus*, and some other poorly characterised rod-shaped or isometric viruses (Forster and Milne 1975, 1978a, b; Fránová et al. 2006; Lee et al. 2003, 2004, 2006a; Lee and Ryu 2006) have been reported.

Among the *Daphne*-infecting viruses, sequence information of the viral genomes is very limited at present. Complete genome sequences of DVS (Lee et al. 2003, 2006b), and DapMV (Fránová et al. 2006) and partial sequence of an isolate of CNSV (Lee and Ryu 2006) have been recently reported, but to date there is no sequence information for the other viruses such as DVX, DVY, and CMV from *Daphne* plants.

In order to obtain molecular information that may help to develop control measures for the viruses, we have determined some sequences of the viruses to develop the molecular detection method in this study. We identified five viruses from collected samples in this study, and all the samples were co-infected with at least three viruses. We report here the incidence of viruses on *Daphne* plants in Korea and the development of molecular detection methods for *Daphne*-infecting viruses from *Daphne* plants in Korea.

Materials and methods

Source of plants used for detection of viruses

In a total of 125 *Daphne* plants (*Daphne odora* vars 'Marginata', 'Zuiko Nikshiki') showing systemic chlorotic spots, mild mosaic systemic stunting with mosaic symptoms, and latent symptoms, were collected from nine nurseries in Seoul and Kyunggi province in 2003–2006. A survey was performed during the late spring to early summer. Some collected plants were maintained in a light/temperature-controlled glasshouse of the Seoul Women's University in Seoul, Korea. All the plants were checked by RT-PCR and some by transmission electron microscopy (TEM) (Lee and Ryu 2006; Yoon and Ryu 2002).

Source of viruses

Five species of *Daphne*-infecting viruses, *Daphne virus Y* (DVY, Potyvirus), *Cucumber mosaic virus* (CMV, Cucumovirus), *Daphne virus S* (DVS, Carlavirus), *Cycas necrotic stunt virus* (CNSV, Nepovirus), and *Watermelon mosaic virus* (WMV, Potyvirus), were originally obtained from the Plant Virus GenBank (http://www.virusbank.org) and used for positive controls for detection of each virus in this study.

Electron microscopy of purified virus preparations

Purified virions from infected leaves of originally infected *Daphne* plant were stained with 2% uranyl acetate and examined with a LEO 906 transmission electron microscope (Yoon and Ryu 2002).

Viral RNA analysis

Viral genomic RNAs were extracted from the purified virus particles by using SDS/proteinase K and phenol extraction method as described by Lee et al. (2003). Viral RNAs of different sizes were separated on a 1.4% denatured agarose gel and visualised by ethidium bromide staining (Sambrook et al. 1989).

Total extraction of RNAs from *Daphne* plants

Total RNAs were extracted from the collected *Daphne* leaf-tissues. All viral genomic RNAs were extracted with SDS-proteinase K/phenol method as described by Lee et al. (2004) and the Rneasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Briefly, each sample of freeze-dried leaf tissues was ground in extraction buffer (50 mM Tris (pH 8.0), 0.1% SDS, 10 mM EDTA) with a sterilised pestle and mortar. Proteinase K (10 mg ml^{-1}) was added to the crude sap preparation and incubated at 42°C for 10 min (Lee and Ryu 2006). The reaction mixture was extracted with phenol-chloroform and total RNAs were ethanol precipitated (Sambrook et al. 1989). The resulting precipitates of total RNAs were dissolved in RNase-free distilled water.

Design of primers for reverse transcription-polymerase chain reaction (RT-PCR)

To design specific-primers for *Daphne*-infecting viruses (CMV, DVY and WMV), cDNAs for individual virus genomic RNAs were generated by a cDNA library kit (Gibco BRL, MD), and resultant cDNA clones were sequenced in this study (Sanger et al. 1977). Nucleotide sequences of DVS and CNSV deposited in GenBank were obtained and used for detection of the viruses. Five sets of detection primers for the viruses were designed based on sequences of CMV, CNSV, DVS, DVY and WMV to amplify 650, 543, 950, 850 and 650 bp, respectively (Table 1).

Table 1 Primers used for detection of *Daphne*-infecting viruses in this study

Virus	Primer name	Sequence (5′–3′)	Region	Size (bp)
DVY	DVYCP5	GAGATAGTACACTATCAGTCC	CP	850
	DVYCP3	AGGTGGTATGACCACTCTTC		
CMV	CMVCP5	ATATGAGCTCTCAGACTGGGAGCA	CP	650
	CMVCP3	ATATGAGCTCATGGACAAATCTGAAT		
DVS	DVSCP5	AAATGCCTCCAAACCAGACCC	CP	950
	DVSCP3	CATTCATTGCTTACGATTATTGGC		
CNSV	CNSVCP5	AGGTATTCTCCATGGTATGG	CP	543
	CNSVCP3	GTAGCAGCATTCGTTGTGCC		
WMV	WMVNIA5	GCC AAA ACA GCA ACG CCA	NIa-Pro	650
	WMVPRO3	AGATGTTAATCCATGTATACC		

RT-PCR detection of five different viruses from *Daphne* plants

Total nucleic acids were extracted from virus-infected leaves as described by Yoon and Ryu (2002), and used as templates for RT-PCR detection. RT was performed in a reaction mixture (20 μl) containing 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 1 μl 50 pM of reverse primer, 1× buffer, 1 U RNasin (Roche, USA), and 2.5 U SSTII reverse transcriptase (Invitrogen, USA) at 42°C for 60 min. PCR was performed in 5 μl of the synthesised cDNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.04 U DNA polymerase Mix *Taq* (Roche, USA), and 1 μl 50 pM of each virus primers. PCR was performed in a thermal cycler (BioRad, USA). Denaturation was executed at 94°C for 3 min before starting PCR cycling. PCR cycle consisted of 30 s at 94°C, 30 s at 52°C, and 40 s at 68°C. A total of 35 cycles was performed, and cycling ended with final extension at 68°C for 10 min.

Results and discussion

Identification of *Daphne*-infecting viruses

Two types of virus particles, filamentous and isometric types, were found in purified preparations from originally infected *Daphne* plants (Fig. 1). Filamentous virus particles are typical of those of carlaviruses and potyviruses, 620 and 780 nm in length, respectively (Fig. 1a), and sequencing analyses demonstrated that carlavirus was identified as DVS and two potyviruses were identified as DVY and WMV. Isometric particles about 30 nm in length were also present in purified preparations (Fig. 1b), and they were identified as

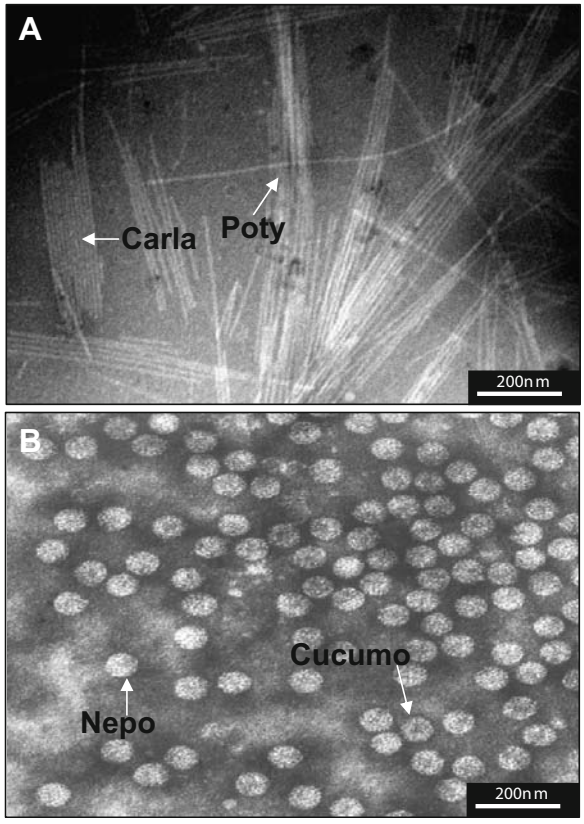

Fig. 1 Electron micrographs of purified virus preparations from *Daphne* plants. Filamentous virus particles (**a**) and isometric virus particles (**b**)

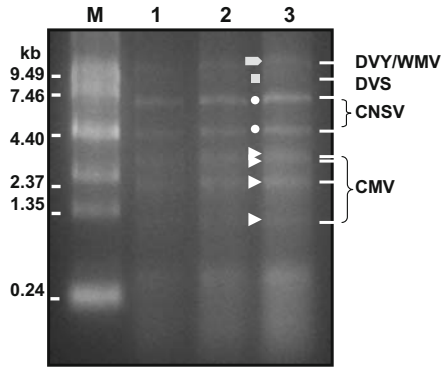

Fig. 2 RNA gel electrophoresis of viral genomic RNAs of five *Daphne*-infecting viruses separated on 1.4% (*w*/*v*) agarose gel. *M* RNA size markers, *1* 1 μg of viral RNAs, *2* 3 μg of viral RNAs, *3* 5 μg of viral RNAs. *Arrowheads* indicate position of genomic RNAs of each viruses

CNSV and CMV by sequence analysis in this study (data not shown). A total of nine RNA bands were observed on gel electrophoresis analysis, and we could identify and differentiate the RNA bands by sizes for genomic RNAs of the five viruses (Fig. 2).

RT-PCR detection of five viruses from *Daphne* plants

In this study, an efficient molecular detection method of *Daphne*-infecting viruses (CMV, CNSV, DVS, DVY and WMV) was developed and applied to the collection of *Daphne* plants. RT-PCR could successfully detect the five viruses by using the RT-PCR from cultivated *D. odora* plants in Korea (Fig. 3), confirmed by partial sequence analyses of each virus. The existence and identification of CMV, DVY and WMV in this study constitute the first report for *Daphne* plants in Korea. To our knowledge, this is the first record of WMV from *Daphne* plants in the world.

High incidence of virus diseases and status of mixed infections of five viruses in *Daphne* plants

Of the 125 *Daphne* plants collected from nine nurseries, incidence of virus infection was >85%. Single virus infection was not detected and all the diseased *Daphne* samples were infected by at least three different viruses. CNSV was the most prevalent

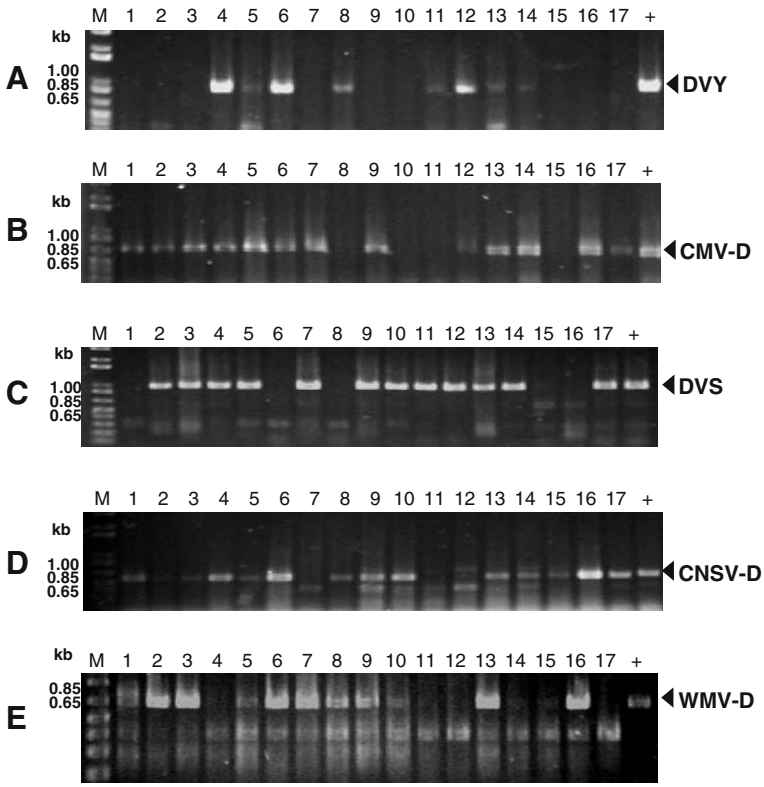

Fig. 3 RT-PCR detection of five *Daphne*-infecting viruses using the virus-specific primers. *Lane M* 1 kb DNA size marker, + positive control, *1–17* individual *D. odora* plants. *Arrowhead* indicates the position of expected RT-PCR products

virus (78%), and the other three viruses such as WMV, CMV and DVS were >60% (Fig. 4a) suggesting virus diseases on *Daphne* plants are extremely severe at present. Especially, it is notable that mixed infections with more than three different viruses were very common (Fig. 4b) and 12% of *Daphne* plants were infected by the five viruses (Fig. 4b). Symptoms on virus-infected *Daphne* spp. included chlorotic spots, systemic warping and distortion on the leaves (data not shown).

Some cultivars of *Daphne* plants have been cultivated in the southern areas and in indoor gardens in Korea. There was currently no report of virus diseases in *Daphne* plants in Korea up until 1999. However, new severe systemic virus-like symptoms have been observed on leaves of cultivated *Daphne* plants (*D. odora*) in Korea since 2000 (Lee et al. 2003; Lee and Ryu 2006). The presence of symptoms significantly influence the quality of plants used for commercialisation, as well as the growth of *Daphne* as a garden plant in Korea. The *Daphne* nurseries in Korea propagate the plants mainly by cuttings (Lee et al. 2003). Therefore, practically, many cultivated *Daphne* plants could be infected with various viruses originating from mother plant sources.

The molecular detection tool described here can be useful for the indexing of the virus from *Daphne* plants to eliminate virus-infected plants during the propagation of mother plants. The infection rate of the virus disease on *Daphne* plants is higher than expected, and therefore careful selection of virus-free plants for propagation is required to protect plants from the pathogenic virus.

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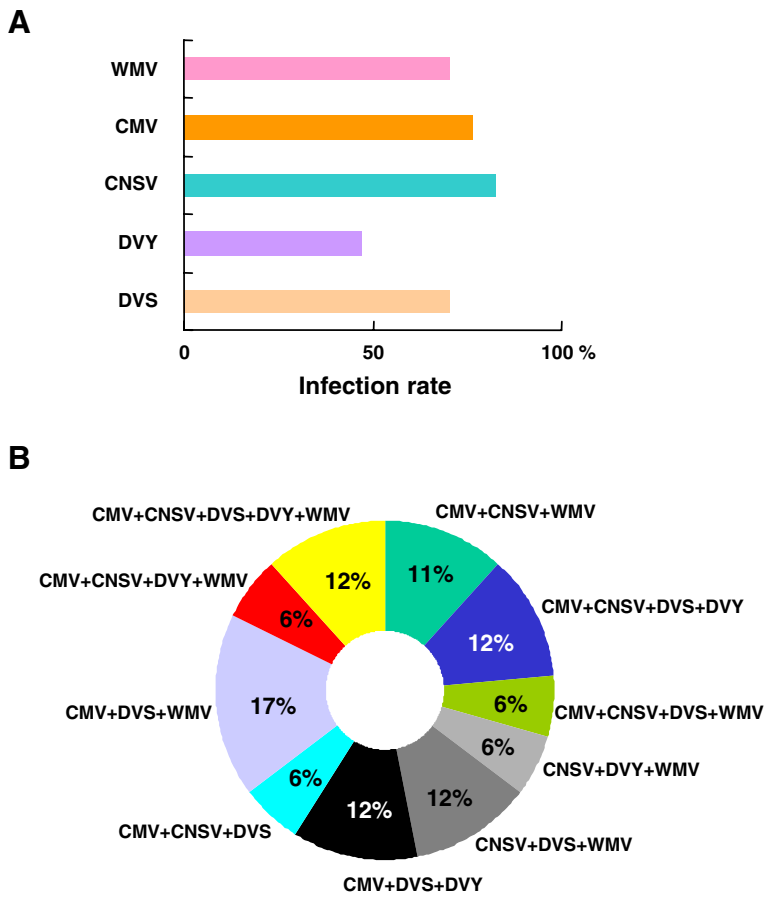

Fig. 4 Infection rate of five *Daphne*-infecting viruses (**a**) and percentages of mixed infections of viruses from *Daphne* plants (**b**)

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