Plant Pathol. J. 22(1) : 63-67 (2006)

The Plant Pathology Journal
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Transmission of *Apple scar skin viroid* by Grafting, Using Contaminated Pruning Equipment, and Planting Infected Seeds

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(Received on December 22, 2005; Accepted on February 15, 2006)

Apple scar skin, one of the most destructive diseases affecting apple, is caused by *Apple scar skin viroid* (ASSVd). Fruit dappling appeared on several cultivars in Korea and has been distributed to major cultivated areas since 2001. ASSVd was identified from infected fruits by using nucleic acid sequence-based amplification with electrochemiluminescence (NASBA-ECL). NASBA-ECL method was faster and hundredfold more sensitive than reverse transcription-polymerase chain reaction (RT-PCR) for ASSVd detection in apple leaves/stems. ASSVd was rapidly transmitted to the entire tree in the second year after artificial inoculation. The ASSVd could be transmitted efficiently by using contaminated pruning scissors to both lignified stems (60 to 70%) and green shoots (20 to 40%) of apple tree and young plants. Dipping of contaminated scissors in 2% sodium hypochlorite solution effectively prevented viroid transmission. In the ASSVd-infected fruits, the viroid was easily detected from fruit skin, seed coat, and embryo. Moreover, embryo and endosperm separately excised from the ASSVd-infected seeds were ASSVd positive in NASBA-ECL assay. Seedlings germinated from ASSVd-positive seeds showed 7.7% infection rate., which indicated that ASSVd is seed-borne.

Keywords : *Apple scar skin viroid*, grafting inoculation, NASBA-ECL, seed-borne, seed transmission

Several viroids are reported to cause significant damages to various crops including fruit trees (Diener 1987; Di Serio et al., 1996; Hashimoto and Koganezawa, 1987; Sano et al., 1989). Apple scar skin, one of the most destructive diseases affecting apple, is caused by *Apple scar skin viroid* (ASSVd) that has been cloned and sequenced (Hashimoto and Koganezawa, 1987; Zhu et al., 1995).

ASSVd belongs to the genus *Apscaviroid* and causes fruit dappling, scarring, or cracking, whereas symptoms do not appear on leaves and stems of the viroid-infected trees. In Korea, ASSVd was first reported in 2001 by Lee et al. (2001). Fruit dappling appeared on several cultivars and has been distributed to major cultivated areas since 2001 (Kwon et al., 2002). A question about natural transmission of ASSVd in orchard was raised because viroid-infected trees have been observed after eradication of infected trees. In this paper, transmission of ASSVd through grafting, seed, and sap inoculation with contaminated pruning scissors was confirmed by NASBA-ECL method.

Materials and Methods

ASSVd isolates. The K1 isolate was collected from 'Chukwang' cultivar showing dappled fruit in Gunwi area, Gyeongbuk province. The isolate was indexed with ELISA to detect the possible presence of *Apple chlorotic leafspot virus* (ACLSV), *Apple stem grooving virus* (ASGV), and *Apple mosaic virus* (ApMV). The isolate was grafted on healthy 'Stark's Earliest' young plant in a glasshouse and used as positive source.

Transmission by grafting with infected scion. Scions infected with ASSVd K1 isolate was top-grafted on 4-year-old 'Fuji/M9' and 'Chukwang/M9' in April 2003. Transmission of ASSVd in treated trees was analyzed from April 2003 to October 2004. Viroid was detected in several parts of the tree as shown in Fig. 1.

Transmission by sap inoculation with pruning scissors. For inoculation, the pruning scissors were contaminated by dipping in crude sap of stem homogenized with 0.01 M phosphate buffer. For inoculation to the mature tree, the 5-year-old trees of 'Hongro/M9' and 'Gamhong/M9' were used for sap inoculation, and 10 cuttings with contaminated scissors in the lignified main stem were accomplished. For inoculation to the young plants, one-year-old 'Chukwang' and 'Stark's Earliest' grafted on seedlings were used as recipient plants, and inoculated with 10 cuts in the one-

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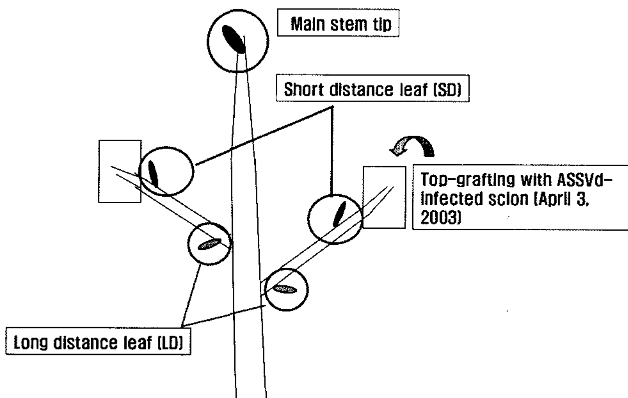


Fig. 1. Tested sample position on the ASSVd-inoculated tree by top grafting.

year-old lignified scion stem or at the base of 4-month-old shoots. The scissors were always decontaminated by dipping in a solution of 2% sodium hypochlorite and drying with absorbent paper. The inoculated cuts were wrapped with parafilm.

Seed transmission. Seeds were extracted from ASSVd-infected fruits. Five fruits were tested viroid infections in fruit skin and seeds. To detect ASSVd, embryo and endosperm were excised from five seeds of viroid-infected fruits with strict disinfection procedure. For seed-borne test, the extracted seeds were treated in low-temperature refrigerator for 50 days to break down seed dormancy. Treated seeds were planted in pots and grown in a glasshouse.

Viroid detection. In graft and sap inoculation transmission test, total nucleic acid was isolated from three leaf discs (about 100 mg) of symptomless young leaf and/or leaf petiole and bark tissues on viroid-inoculated apple trees by silica-based extraction method (Boom et al., 1990) with NucliSens automatic extractor (BioMeruix Co., France). For ASSVd detection from seeds, total nucleic acid was isolated from embryos or endosperms excised from five seeds. Viroid detection was performed by using NASBA-ECL method (Kim et al., 2004). Design of the ASSVd-specific primers, P1 and P2 for amplification of the viral RNA by NASBA was based on the previously reported sequences (Lee et al., 2001) and amplified a 251 bp fragment of ASSVd gene. These primers were designed and labeled with the T7 RNA polymerase promoter and ECL detection sequences, respectively. And Biotin-labelled specific capture probe was designed complementary to the internal nucleotide sequence of the ASSVd. NASBA reactions used 5 μl of purified viral RNA, a 0.2 μM primer concentration, and 70 mM KCl, with reactions performed according to the NucliSense Basic Kit instructions (Bio-Meruix Co. France). Briefly, 10 μl of the premix cocktail was dispensed into thin-walled Eppendorf tubes and 5 μl of nucleic acid eluate was added to each tube. The tubes were preheated at at 94°C for 2 min and incubated at 65°C for 5 min followed by cooling to 41°C for 5 min. A 5 μl aliquot of enzyme mix (RNA polymerase, RNase H, and AMV-reverse transcriptase) was added to each tube followed by incubation for 120 min at 41°C. The NASBA-generated products were detected by liquid hybridization using the ECL principle employed by the NucliSens reader (Bio-Meruix Co., France). Amplification product (5 μl) were diluted in 100 μl of detection buffer provided with the NucliSens Basic Kit. Hybridization was carried out using 20 μl of a 1:1 mixture of streptavidin-coated capture probes coupled to magnetic beads and Ru^{2+}-labeled oligonucleotide detection probes, and 5 μl of the 1:20 diluted amplification product. The solution was incubated at 41°C for 30 min after which assay buffer (300 μl) was added to the tubes followed by detection using the NucliSens ECL reader. After incubation, the paramagnetic beads carrying the hybridized amplicon/ECL probe complexes were magnetically captured on the surface of an electrode that is part of the ECL reader. Voltage applied to the electrode triggers the ECL reaction, such that the light emitted by the hybridized Ru^{2+}-labeled probes is proportional to the quantity of amplicon present.

Results

ASSVd was rapidly transmitted to adult trees inoculated by top grafting with infected scion. The viroid was detected in leaves that are next budding from grafting union 60 or 90 days after inoculation (data not shown). In its first year of inoculation, ASSVd was not detected from the leaves of 'Chukwang' cultivar positioned away from grafting union. In 'Fuji' cultivar, however, the leaves positioned away from the grafting union ASSVd was detected 120 days after inoculation (Table 1). The artificially inoculated viroid was detected from all parts of sprouting and harvest time in second year. Furthermore, the fruits showed dappling in 'Fuji' cultivar but fruits on 'Chukwang' cultivar had no dapple symptom.

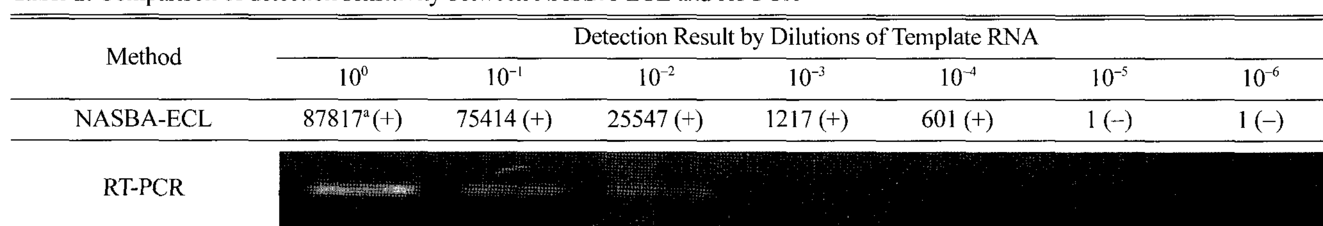
The NASBA-ECL method was faster and hundredfold more sensitive than RT-PCR for ASSVd detection in apple leaves (Table 2). ASSVd was easily transmitted by using contaminated pruning scissors (Table 3 and 4). ASSVd was transmitted efficiently with contaminated scissors to both lignified stems (60%-70%) and green shoots (20%-40%) of apple tree and young plants when they were assayed with NASBA-ECL methods. Dipping of contaminated scissors in 2% sodium hypochlorite solution effectively prevented

Table 1. Viroid transmission on ASSVd-inoculated trees by top grafting

Cultivars	Detection Time	NASBA-ECL Detection Result							
		Leaf position						Fruits	
		GS[a]	SD	LD	TMS	MMS	BMS	skin	symptom
Fuji	Aug. 2003	+[b]	+	+	–	nt	nt	nt	No
	June 2004	+	+	+	+	+	+	nt	
	Oct. 2004	+	+	+	+	+	+	+	Yes
Chukwang	Aug. 2003	+	+	–	–	nt	nt	nt	No
	June 2004	+	+	+	+	+	+	nt	
	Sept. 2004	+	+	+	+	+	+	+	No

[a]GS, grafting scion; SD, short distance leaf from grafting union; LD, long distance leaf from grafting union; TMS, top leaf of the main stem; MMS, middle leaf of the main stem; BMS, base leaf of the main stem;
[b]+, positive reaction ; –, negative reaction; nt, not tested.

Table 2. Comparison of detection sensitivity between NASBA-ECL and RT-PCR

Method	Detection Result by Dilutions of Template RNA						
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
NASBA-ECL	87817[a](+)	75414 (+)	25547 (+)	1217 (+)	601 (+)	1 (–)	1 (–)
RT-PCR							

[a]ECL signal indicated.

Table 3. Transmission rate of ASSVd from naturally infected stems to lignified healthy plant by using contaminated pruning scissors

Plants	Tested Plants	Number of ASSVd Positive Plants by Day After Inoculation						Transmission	
		30 DAI	60 DAI	90 DAI	120 DAI	180 DAI	210 DAI	Positive plants	%
Tree	5	–	0	0	1	2	3	3	60
Plants	10	0	5	6	7	–	–	7	70

Table 4. Transmission of ASSVd from naturally infected stems to green shoot of healthy plant by using contaminated pruning scissors

Plants	Inoculation	No. of Test Plants	NASBA-ECL Positive Plants		Transmission	
			Leaf	Fruit	Positive	%
Tree	5 cuts with contaminated scissors	5	0	2	2	40
	5 cuts and dipped in 2% NaOCl solution	10	0	0	0	0
Plantlets	5 cuts with contaminated scissors	10	2	–	2	20
	5 cuts and dipped in 2% NaOCl solution	7	0	–	0	0

Table 5. Viroid detection of ASSVd-infected fruit and its seeds

Fruits	NASBA-ECL Signal (× 1000)[a]		
	Fruit skin	Seed coat	Embryo
Ck1	437 (+++)	257 (+++)	12 (+)
Hh1	11 (+)	0.1 (–)	0.1 (–)
Hh2	14 (+)	66 (+)	0.03 (–)
Fj1	495 (+++)	433 (+++)	326 (+++)
Fj2	460 (+++)	273 (+++)	11 (+)

[a]Calculated signal was described, and cut off level was 0.3.

viroid transmission (Table 4).

In the ASSVd-infected fruits, the viroid was easily detected from fruit skin, seed coat, and embryos (Table 5). The degree of infection in embryo was proportional to the viroid concentration in the fruit. Moreover, embryo and endosperm separately excised from the ASSVd-infected seeds were positive in NASBA-ECL assay (Table 6). Transmission through seed was also examined and seedlings germinated from ASSVd-positive seeds showed 7.7% infection rate (Table 7).

Table 6. Viroid detection on embryos and endosperm excised from ASSVd-infected 'Fuji' seeds

Detected Tissue	NASBA-ECL Reaction by Experiment				
	1st	2nd	3rd	4th	5th
Embryo	–	++	+	–	+
Endosperm	++	++	nt[a]	nt	nt

[a]nt, not tested.

Table 7. ASSVd detection on seedlings germinated from viroid-infected seeds

Detected Seedlings	Positive Plants	Transmission Rate (%)
90	7	7.7

Discussions

This study showed that ASSVd could be transmitted by contaminated scissors and planting infected seeds. Also, ASSVd is easily transmitted by grafting. In an earlier experiment Koganezawa (1985) reported that ASSVd could be transmitted by razor splashing. This study, however, suggests that the viroid may be easily transmitted in orchards from infected to healthy trees by contaminated pruning equipment. Peach latent mosaic viroid (PLMVd), Citrus exocortis, and other citrus viroids also have been reported to be transmitted through contaminated tools (Hadidi et al., 1996; Roistacher et al., 1969; Kyriakou, 1992). Hadidi et al. (1991) previously reported scar skin and dapple apple viroids are seed-borne. However, vertical transmission of ASSVd from seed to seedling has not been elucidated yet. Several researchers showed that ASSVd is seed-borne but they could not confirm the seed-transmission of ASSVd (Desvignes et al., 1999; Howell et al., 1998; Hurtts and Podleckis, 1995). ASSVd has been detected in whole seeds from infected pome fruits, particularly in the seed coats and subcoat and embryo and endosperm, which suggested the possibility of seed transmission of ASSVd (Wallace and Drake, 1962; KryczyZski et al., 1988). Moreover, our observation indicates that this viroid is seed-transmitted. This is a different result from the previous reports showing that ASSVd is not seed-transmitted. Wah and Symons (1999) previously reported transmission of grapevine viroid via seed from in vitro germination of grape seeds. The seedlings used in this experiment were sterilized with disinfection procedure and germinated with strict growth condition in glasshouse. Thus, we consider that other transmission factor was impossible. Dodds et al reported that the transmission through seed collected from symptomatic trees is low (less than 5%) but is reported to be high (80 to 100%) from symptomless carriers. This is very similar result with our study.

In this paper, the seed-transmission of ASSVd was proved by using sensitive detection method, NASBA-ECL. NASBA, nucleic acid sequence-based amplification, is an isothermal technique with a broad potential application in RNA amplification and detection (Sooknanan et al., 1995). NASBA is based on the concurrent activity of avian myeloblatosis reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase, together with two primers to produce the amplification (Kievits et al., 1991). These characteristics of NASBA make the method particularly appropriate for high-throughput sample analysis and for development of completely automated workstations (Sooknanan et al., 1995). This method was more sensitive than RT-PCR. Several studies have also demonstrated that NASBA is more sensitive than RT-PCR (Goossens et al., 2000; Greene et al., 2003; Lunel et al., 1999).

This study showed that ASSVd is vertical transmitted via seed and horizontal transmitted via contaminated scissors. Both transmission routes, particularly the second, explain the persistence of ASSVd in nature. Therefore, using viroid-free sources of seed, seedlings, rootstocks, and budwood should greatly reduce the risk of future spread of the viroid.

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