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Note

Effective Application of CF11 Cellulose for Detection of *Apple scar skin viroid* in Apple

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The low virus titer in woody plant tissues and the presence of inhibitor compounds such as polyphenols, tannins and polysaccharides are common difficulties that compromise purification of plant viroids from their woody hosts. A simple, reliable method of RNA isolation using CF11 cellulose column on a microcentrifuge tube scale for detecting *Apple scar skin viroid* (ASSVd) in apple was developed. Total RNA extracted from leaf, woody bark and the fruit skin was used for reverse transcription. RT-PCR products could be detected from RNA prepared from dormant woody bark, fruit skin and fresh leaves with both the CF11 cellulose column method and NucliSens extractor in February, August and November. Meanwhile, with the RNeasy kit RT-PCR, products were detected only in leaves and not from bark or fruit skin. The PCR product, about 330 base pairs, was analyzed by agarose gel electrophoresis. The CF11 cellulose column method was effective for detecting ASSVd. The method enabled the processing of a large numbers of samples of dormant woody bark, leaf and fruit skin of apple.

Keywords : ASSVd, apple, CF11, detection, RT-PCR, viroid

Viroids are single-stranded circular RNA molecules that cause serious diseases in economically important fruit trees (citrus, apple, peach, grape, coconut and avocado). *Apple scar skin viroid* (ASSVd) has been found to occur in apple in Korea (Lee et al., 2001). The viroid affects the quality of apple fruit by causing dapple symptoms on the skin (Fig. 1) (Koganezawa et al., 1983; Lee et al., 2001). For the sensitive detection of ASSVd in apple, a simple and reliable extraction procedure of nucleic acids is required.

Often it is difficult to extract relatively pure nucleic acids from some plants for detection of viruses or viroids. For example, the low titer in woody plant tissues (Hu et al., 1990; Zee et al., 1987) and the presence of inhibitor compounds such as polyphenols, tannins and polysaccharides are common difficulties that compromise purification of plant viruses and viroids from their woody host plants (Ling et al., 2000).

The previously published nucleic acid extraction procedure for detection of ASSVd in apple was a silica-based extraction method using an expensive apparatus, the NucliSens automatic extractor (BioMeruix Co., France) (Kim et al., 2004). Moreover, extraction with organic solvents followed by using an expensive commercial minicolumn (Qiagen kit) was used (Lee et al., 2001).

CF11 cellulose can be used for isolation of RNA from plants like chrysanthemum which contains high amount of phenolic compounds and polysaccharides, because it has the ability to bind RNA in the presence of the appropriate concentration of ethanol. This provided a convenient way to assess plants for the presence of dsRNA (Morris and Dodds, 1979). CF11 cellulose was used for detection of *Chrysanthemum stunt viroid* (CSVd) in chrysanthemum (Chung et al., 2006; Shiwaku et al., 1996).

In this study, a CF11 cellulose column was used for detection of ASSVd in apple in comparison to the NucliSens RNA extractor and the Qiagen RNeasy kit.

Source of ASSVd-infected apple. Two ASSVd-infected

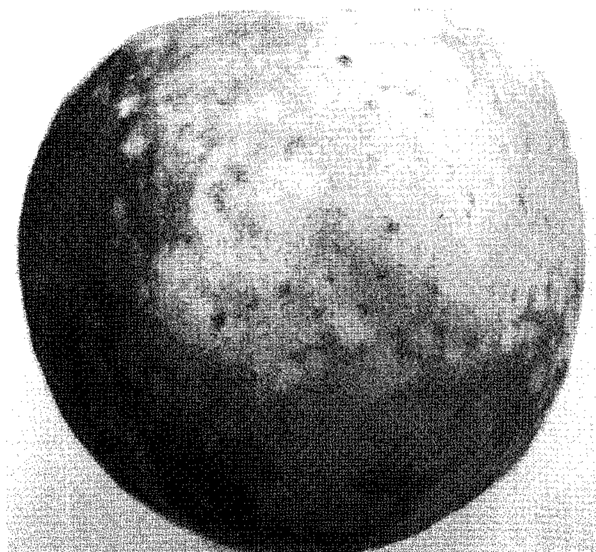

Fig. 1. Dapple symptom caused by infection with *Apple scar skin viroid* in apple (*Malus pumila*) cv. Fuji.

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apple trees, which are maintained in an experiment plot at the NIHHS in Suwon, were used as the source of ASSVd. The plant tissues tested for extraction of RNA were bark in February, leaf and bark in August and leaf, bark and fruit skin in November.

RNA extraction methods. RNA was prepared using a CF11 cellulose (Whatman) column, NucliSens RNA extractor, or RNeasy kit (Qiagen), as described below. In RNeasy kit and NucliSens extractor methods, RNA was prepared with 0.1 g of plant tissues according to the manufacturers' instructions. In the CF11 cellulose column method, the procedure of Shiwaku (1996) was modified and shortened. About 0.1 g of plant tissue, powdered in liquid nitrogen, was suspended in 600 μl of extraction buffer (0.1 M Tris-HCl, 50 mM EDTA, 2.8 M NaCl, pH 8.0) followed by adding 60 μl 20% SDS, 0.05 g polyvinylpyrrolidone, 1 μl 2-mercaptoethanol and 600 μl of phenol:chloroform (1:1). One ml of supernatant was taken from the extract, after centrifugation at 13,000 rpm for 13 min, and incubated for 10 min at 65 °C. Four hundred μl of 5 M potassium acetate was added to the extract, which was then placed on ice for 20 min. And then 600 μl of ethanol was added to the extract, which was then applied to 0.05 g of cellulose column saturated in 30% ethanol in 1× STE buffer (50 mM Tris-HCl, pH 7.2, 1 mM EDTA, 100 mM NaCl). After centrifugation of the extract mixed with CF11 cellulose, the column was washed three times with a 1× STE buffer including 30% ethanol. RNA was eluted from the column with 500 μl of 1× STE buffer after centrifugation at 13,000 rpm for 10 min followed by ethanol precipitation of the eluate.

Reverse transcription and polymerase chain reaction (RT-PCR). A pair of primers was designed on the basis of the ASSVd sequence (GenBank accession no. EU825613). The sequence of the forward primer was homologous to nucleotides 102 to 115 (5'-GGTAAACACCGTGCGGTT-CCT-3') of the ASSVd isolate and the sequence of reverse primer was complementary to nucleotides 162 to 184 (5'-GGGAAACACCTATTGTGATTTACC-3') of the ASSVd isolate.

Complementary DNA (cDNA) synthesis was accomplished as follows: Ten ng of RNA in 9 μl of nuclease free water and 1 μl of 10 pm reverse primer was heated at 70 °C for 5 min followed by adding 4 μl of 5× reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μl of ImProm II reverse transcriptase (Promega, USA) and 1 μl of RNase inhibitor (1 U/μl) on ice, and incubating at 37 °C for 1 hr. PCR amplification was performed in a 50 μl volume containing 20 μl of the cDNA solution, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1 μl of 10 pm each primer, 2.5 units of DNA polymerase (Promega, USA), and the 1× PCR buffer. Forty PCR cycles were conducted in PTC-0220 Perlitier Thermal Cycler (MJ Research, MA, USA). The thermal conditions were as follows: denaturation at 94 °C for 30 sec (2 min for the first cycle), annealing at 51 °C for 40 sec and extension at 72 °C for 1 min. The final extension was for 10 min.

RT-PCR products could be detected from RNA prepared from dormant woody bark, fruit skin and fresh leaves, with both the CF11 cellulose column method and NucliSens extractor in February, August and November (Fig. 2). Meanwhile, with the RNeasy kit RT-PCR products were detected only in leaves and not from either bark or fruit skin (Fig. 2).

The greatest advantage of this CF11 cellulose column method is that it does not require expensive equipment or commercially prepared columns. The time required for preparation of RNA using the CF11 cellulose column method is 4 hrs for processing 24 samples. The NucliSens extractor method requires a similar time for the same number of samples. However, the NucliSens extractor method is very expensive in comparison to the CF11 cellulose column method, the running costs of which are only 10% of the former method, excluding the cost of the NucliSens apparatus itself (about 110,000 US dollars). In addition, this CF11 cellulose column method is simple on a microcentrifuge tube scale and allows a large number of samples to be processed at one time. In a previous report (Branch et al., 1988), CF11 cellulose chromatography was applied to extract *Potato spindle tuber viroid* from tomato. In those

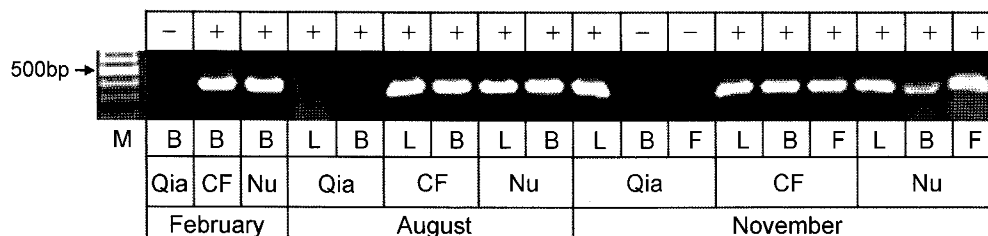

Fig. 2. Agarose gel electrophoresis of RT-PCR products of 330 base pairs, amplified from apple infected with *Apple scar skin viroid.* RNAs were extracted using the RNeasy kit (Qiagen) (Qia), CF11 cellulose column (CF) and NucliSens extractor (Nu) in leaf (L), woody bark (B) and fruit skin (F) collected in February, August and November.

experiments, they extracted dsRNA from 1 g of plant tissues using a 10 ml CF11 cellulose column and rechromatographed on two successive 2 ml columns of CF11 cellulose. This is comparable in column size with this present study, since we used only a 2 ml tube for the CF11 cellulose column.

Low virus titers and the presence of inhibitor compounds such as polyphenols, tannins and polysaccharides present in woody plants make the detection of viruses or viroids difficult (Hu et al., 1990; Ling et al., 2000; Zee et al., 1987). CF11 cellulose should be applicable to the detection of viruses or viroids, including *Apple dimple fruit viroid, Citrus exocortis viroid, Peach latent mosaic viroid, Grapevine yellow speckle viroid, Coconut cadang-cadang viroid* and *Avocado sunblotch viroid*, from their natural woody hosts apple, citrus, peach, grape, coconut and avocado, respectively.

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