RAPID ISOLATION AND SENSITIVE DETECTION OF THE CHRYSANTHEMUM CHLOROTIC MOTTLE VIROID

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ABSTRACT

Chrysanthemum chlorotic mottle viroid (CCMV) is one of two viroids which infect chrysanthemum (*Dendranthema grandiflorum*). These viroids are the smallest known agents causing infectious disease. The pathogens are small (246-401 nucleotides), circular, single-stranded RNAs that do not code for any protein. CCMV infections of chrysanthemum are a severe problem throughout the world. However, it is difficult to detect the pathogen in plant tissues due to low levels of pathogen being present. In addition, there may be substances in the samples which are inhibitory to sensitive downstream detection assays. For these reasons, Norgen has developed a Chrysanthemum Chlorotic Mottle Viroid RT-PCR Detection Kit. This kit is a ready-to-use system for the isolation and detection of CCMV directly from plant samples. The kit contains components for the rapid isolation of total RNA, including viroid RNA, which is free from inhibitors, using spin-column chromatography based on Norgen's proprietary resin. In addition, the kit contains a CCMV Master Mix and controls to allow for PCR amplification of the viroid. The CCMV Master Mix contains reagents and enzymes for the specific amplification of a 346 bp region of the viroid genome. In addition, Norgen's Chrysanthemum Chlorotic Mottle Viroid RT-PCR Detection Kit contains a second heterologous amplification system to identify possible PCR inhibition and/or inadequate isolation. Amplified PCR products are then detected using agarose gel electrophoresis. The amplification bands are clearly distinct allowing for easy assay interpretation. The kit is highly sensitive and specific with a limit of detection of 100 genome copies. This kit will be invaluable not only to plant molecular biologists but also to commercial propagators in providing the Chrysanthemum industry with plant material that is free of infectious agents.

INTRODUCTION

Chrysanthemum chlorotic mottle viroid (CCMV) is one of two viroids that infect chrysanthemum (*Dendranthema grandiflorum*). CCMV infections of many chrysanthemum cultivars have been a severe problem in the world. Although the magnitude of the infection of chrysanthemum plants varies between cultivars, general symptoms are as follows: 1) young leaves become light green; 2) plant height decreases (stunting); 3) the anthocyanin in stems disappears; 4) leaves and flowers become small and; 5) the rooting ability decreases. It is very difficult to produce CCMV-free chrysanthemum plants. Highly sensitive detection methods using reverse transcription polymerase chain reaction (RT-PCR) of CCMV infected chrysanthemum plants have recently progressed. However, the preparation of RT-PCR templates requires laborious steps for eliminating polyphenols and polysaccharides, both of which are inhibitory to the PCR detection. In addition, a large amount of sample is needed for the RNA template preparation thus; CCMV detection and the screening for CCMV-free plants can be carried out only once the *in vitro*-cultured plants are grown to the desired size for the template preparation. This step makes the CCMV-free plant production more difficult. In plant science, some methods for RNA template preparation have been established. However, these methods do not totally remove the inhibitory compounds to PCR and false negative results are often observed. Methods to isolate the CCMV RNA from plant tissue that is free of such inhibitors are therefore required. The purified RNA can then be successfully employed in sensitive downstream detection assays such as RT-PCR and RT-qPCR. This research initiative developed a method for the isolation of inhibitor-free RNA from plants, which could successfully be used in RT-PCR for the detection of CCMV.

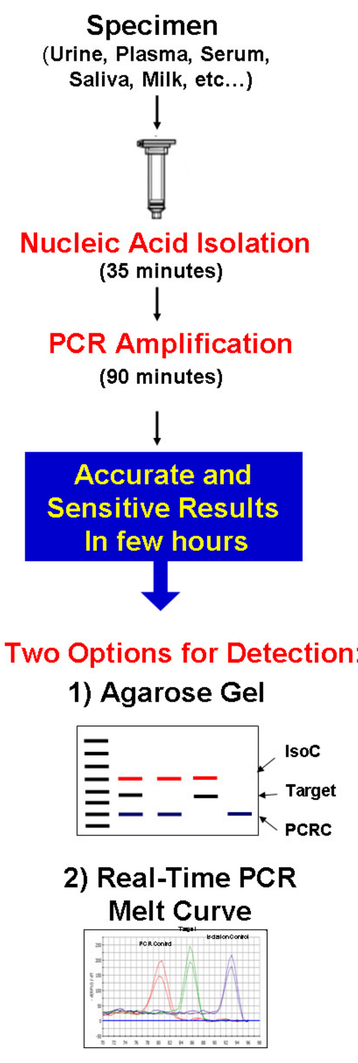

Figure 1. Norgen's CCMV RT-PCR Detection Kit is a ready-to-use system for the isolation and detection of CCMV from plant samples. First, the kit contains components for the rapid isolation of total RNA, including viroid RNA, from the samples using spin-column chromatography based on Norgen's proprietary resin. Second, the kit contains CCMV Master Mix and controls to allow for PCR Amplification. The amplified PCR products are then detected using agarose gel electrophoresis. Alternatively, detection can be performed based on real-time PCR using melt curves.

RESULTS

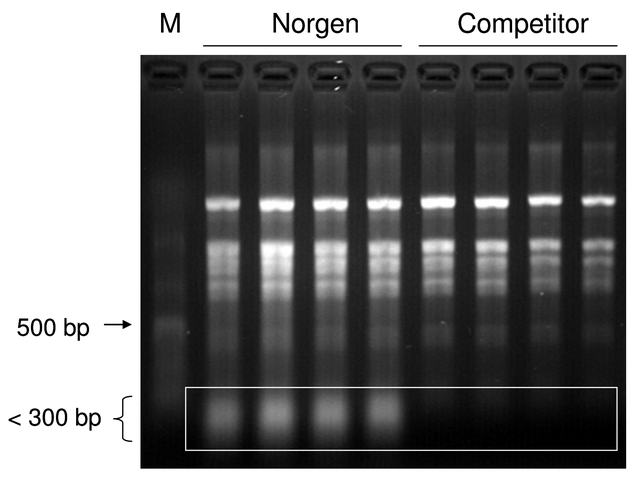

Figure 2. Efficient Isolation of Total RNA, including Small RNA. Total RNA was isolated from 50 mg of Chrysanthemum leaves using Norgen's Viroid RNA Purification Kit, which uses the same RNA isolation technology as Norgen's CCMV RT-PCR Detection Kit, and a leading competitor's kit. The RNA profiles were then compared by visualizing the RNA on a 1.2% formaldehyde gel. Small RNA under 300 bp, the size of the most common Viroid, was predominantly obtained from the Norgen's Viroid RNA Purification Kit, while the competitor did not successfully isolate the small RNA. For visualization, 7.5 uL of each 50 uL elution was loaded on the 1.2 % formaldehyde RNA gel. Lane M is Norgen's 100 b RNA Ladder.

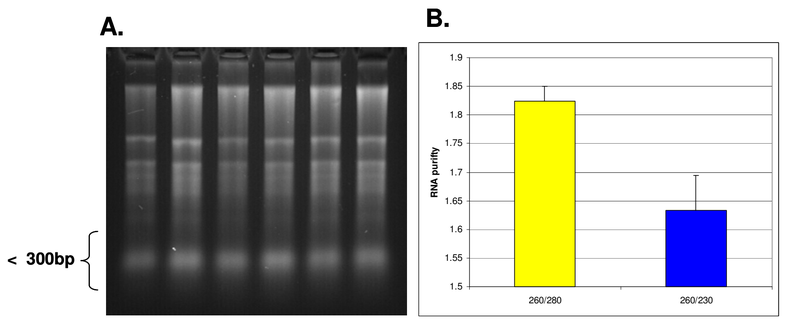

Figure 3. Efficient Isolation of High Quality Total RNA, including Small RNA. Total RNA was isolated from 50 mg of Chrysanthemum leaves using Norgen's CCMV RT-PCR Detection Kit. In Panel A the RNA profiles were visualized on a 1.2% formaldehyde gel. Small RNA under 300 bp, which corresponds to the size of CCMV RNA, was obtained from all the samples tested. For visualization, 7.5 µL of each 50 µL elution was loaded on the 1.2 % formaldehyde RNA gel. Lane M is Norgen's 100 b RNA Ladder (Cat. 15002). Average total RNA yield was 8.7 µg from 50 mg of leave tissue. In Panel B 2 µL of eluted total RNA was analyzed by the NanoVue Plus™ (GE Healthcare) according to the manufactures manual. The result shows that the purified total RNA has a superb quality for down stream applications such as RT-PCR to detect CCMV.

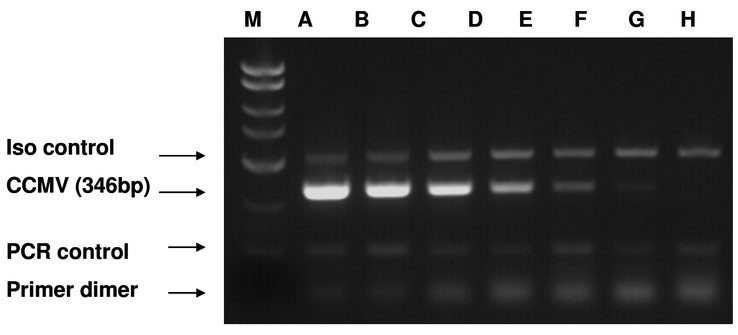

Figure 4. Sensitivity of Detection using the CCMV RT-PCR Detection Kit. A representative 1X TAE, 1.7% agarose gel showing the amplification of CCMV at different concentrations (Target) by one-step RT-PCR. The size of the CCMV target amplicon corresponds to the 346 bp band represented by the provided DNA Marker (M). The size of the Isolation Control corresponds to the 520 bp band represented by the provided DNA Marker (M). The CCMV 2X PCR Master Mix contains a PCR Control which controls for PCR inhibition. The size of the PCR Control corresponds to the 150 bp band represented by the provided DNA Marker (M). Lanes A- G represents samples spiked with different concentration of CCMV transcript expressed in *E.coli* (interpreted as positive results). Lane H represents a no CCMV control.

Figure 5: Detection of CCMV using the CCMV Detection Kit. A representative 1X TAE 1.7% agarose gel showing the amplification of CCMV at different concentrations (CCMV Target). The size of the CCMV target amplicon corresponds to 346 bp as represented by the provided RNA Marker (M). The size of the CCMV Isolation Control (Iso C) corresponds to 520 bp as represented by the provided RNA Marker (M). The CCMV 2X PCR Master Mix contains a CCMV PCR Control (PCRC). The CCMV PCRC controls for PCR inhibition. The size of the CCMV PCRC corresponds to 150 bp as represented by the provided RNA Marker (M). The amplification from each lane is interpreted as shown below. Lane 1 to 4: CCMV detected as all Lanes showed the detection of all three PCR amplicons. Lane 5: No IsoC and Template– Only CCMV PCRC was detected. Lane 6: CCMV not detected: Detection of CCMV IsoC and CCMV PCRC, suggesting that the RNA isolation was successful but no CCMV RNA was present in the sample. Lane 7: Positive: All three PCR amplicons were detected

Table 1. Norgen Biotek Viroid RNA Purification and Detection Kits

Kit	Description
Viroid RNA Purification Kit	Isolate high quality viroid RNA from a wide range of plant species Isolate all sizes of viroid RNA without phenol No liquid nitrogen required for homogenization Fast and easy processing Available in spin column format and 96-well format for high throughput applications
Chrysanthemum Chlorotic Mottle Viroid RT-PCR Detection	Isolation and detection of CCMV from plant tissues.
Potato Spindle Tuber Viroid RT-PCR Detection Kit	Isolation and Detection of Potato Spindle Tuber Viroid from plant tissues.
Hop Latent Viroid RT-PCR Detection Kit	Isolation and Detection of Hop Latent Viroid from plant tissues.
Avocado Sunblotch Viroid RT-PCR Detection Kit	Isolation and Detection of Avocado Sunblotch Viroid from plant tissues.
Hop Stunt Viroid RT-PCR Detection Kit	Isolation and detection of Hop Stunt Viroid from plant tissues.
Tomato Chorotic Dwarf Viroid RT-PCR Detection Kit	Isolation and Detection of Tomato Chlorotic Dwarf Viroid from plant tissues.
Chrysanthemum Stunt Viroid RT-PCR Detection Kit	Isolation and detection of Chrysanthemum Stunt Viroid from plant tissues.

Discussion

The results of this study indicate that RNA extraction from plant samples with Norgen's proprietary resin yields high quality, inhibitor-free RNA, which can be successfully amplified by RT-PCR for the detection of CCMV. To detect viroids using PCR it is important that the samples of nucleic acids be as pure as possible. This is especially true when a reverse transcription phase is necessary, as the reverse transcriptase is highly susceptible to interfering or inhibitory substances. Norgen's CCMV RT-PCR Detection Kit is an efficient, simple and reproducible method for the isolation of viroid RNA from infected plant tissues. The rapid procedure allows the isolation and detection of CCMV in under 3 hours. The isolated RNA was of high quality and could serve as a robust template for reverse transcription. Under conditions of the CCMV RNA isolation procedure, Norgen's CCMV RT-PCR Detection kit can detect viroid in the linear range of 100 copies up to 1 x 10^6 copies, indicating the high sensitivity and specificity of the method. As the resulting amplification bands are clearly distinct it also offers accuracy and ease of interpreting results. This kit will be invaluable to both plant molecular biologists who study viroids as well as commercial plant propagators who wish to supply the Chrysanthemum industry with plant materials and seeds which are free of infectious agents.

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