A New Gold Standard for Viroid Detection; Comparison Study Between Membrane Hybridization and RT-PCR

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Abstract

A one-step RT-PCR based assay is compared to the membrane hybridization assay (Agdia, Elkhart, IN) for the detection of the Chrysanthemum stunt viroid (CSVd) using purified RNA from chrysanthemum as the template/input. Based on the sensitivity, specificity, positive predictive value, and negative predictive value, the one-step RT-PCR was found to be a much more efficient CSVd detection method than membrane hybridization. The primers used in this study were shown to be highly specific with no cross reaction to 9 other viroids tested. This compares to membrane hybridization, which was found to have a 61% false-positive rate from the same samples. The Norgen RNA extraction kit and primer set were shown to provide highly efficient extraction of small molecular weight RNA's and ideally suited to viroid isolation and assay. The sensitivity of the membrane hybridization method was found to be 73%, with a detection limit of 1 x 107 copy number. This compares to the one-step RT-PCR method, which had 100% sensitivity, with a detection limit reaching as low as only a few transcript copies (i.e. 1 x 106 more sensitive). The availability of a one-step RT-PCR assay provides a new rapid test for the screening of chrysanthemum cultivars to reduce losses caused by CSVd in the industry.

Introduction

- First described in 1945 in the United States[1], Chrysanthemum stunt viroid (CSVd) has spread throughout greenhouses within both the USA and Canada with infection rates as high as 50-100%.
- Symptoms of chrysanthemum stunt are often difficult to detect. Flowering plants often have shortened stems, premature or uneven flowering, reduced flower size and bleaching in pigmented flowers. These symptoms can be visualized in Figure 1. Foliar symptoms are less common, but may be seen as smaller, paler leaves, sometimes having leaf spots or flecks[2].
- Since many plants can remain symptomless until flowering, infection can spread throughout greenhouses before the problem becomes evident. A method for the detection of CSVd before symptoms appear is therefore required to prevent the spread of this viroid to healthy plants.
- Agdia Diagnostic Services (Elkhart, IN.) offers a detection service based on the hybridization assay, and a ready-to-use kit for the isolation and detection of CSVd using end-point PCR is available commercially through Norgen Biotek Corp. (Thorold, ON).
- This poster will compare the two common methods of CSVd detection: membrane hybridization and one-step RT-PCR. Membrane hybridization requires 3µl of sample, and results can be obtained in 20 hours. One-step RT-PCR requires only 2µl of sample, with results obtained in a mere 3 hours.

Objective of the Study

To compare two common CSVd detection methods: the hybridization assay (Agdia), and the end-point RT-PCR method (Norgen Biotek). These methods will be compared and contrasted based on their overall sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV) (calculated using other common viroids to thoroughly study each method's cross-reactivity).

Materials and Methods

Total viroid RNA purification. Total RNA was purified from 0.5 g of frozen leaf or bud tissue from healthy and infected chrysanthemum plants using the Viroid RNA Purification Kit (Norgen Biotek Corp., Thorold, ON). *Chrysanthemum x moriflorium* L. cv Shamrock was used to develop the one-step RT-PCR method. Chrysanthemum cvs. Chesapeake, Durango, Juneau, Icecap, Puebla, Puna, Pelee, Shamrock, Snowball, and Viron were also used in this study.

CSVd in vitro transcription and quantification. A partial CSVd nucleotide sequence (354 bp) from NCBI (Ref. No. FN673554) was synthesized and cloned into pIDBlue (IDT, IA). The plasmid was linearized at the EcoRI site and used as the target in an in vitro transcription reaction performed with Megascript T7 kit (Ambion Inc., TX). DNase I-treated RNA was cleaned and concentrated using the RNA Clean-Up and Concentration Kit (Norgen Biotek, ON). The amount of RNA in grams (g) was then quantified by NanoVue Plus Spectrophotometer (GE Healthcare, QC). The CSVd copy number was then calculated using web-based software (http://www.endmemo.com/bio/dnacopynum.php). The number of transcripts was calculated per 2 µl, which was the volume used as the template in a one-step RT-PCR. Ten-fold serial dilutions of the transcripts were prepared from 5.42×10^{12} to 5.42×10^{1}. The one-step RT-PCR was carried out in an iCycler IQ (Bio-Rad). For membrane hybridizaton, 3 µl of all samples were blotted on the hybrid membrane, as per manufacturer's protocol.

Specificity of detection. The specificity of the CSVd one-step RT-PCR primer set was tested against 9 different viroids: 1) Chrysanthemum chlorotic mottle viroid (CChMVd); 2) Potato spindle tuber viroid (PSTVd); 3) Avocado sunblotch viroid (ASBVd); 4) Tomato chlorotic dwarf viroid (TCDVd); 5) Hop stunt viroid (HSVd); 6) Citrus exocortis viroid (CEVd); 7) Coconut cadang-cadang viroid (CCCVd); 8) Apple scar skin viroid (ASSVd); and 9) Tomato Planta Macho Viroid (TPMVd). One-step RT-PCR reactions were performed as above for the detection of the various viroids and compared to the detection of CSVd. Similarly, the same viroids were spotted randomly on the hybrid membrane.

Detection of CSVd in different inoculated chrysanthemum cultivars. In the first trial, 2-4 plants of 5 chrysanthemum cvs. were obtained from a commercial chrysanthemum grower with a high incidence of CSVd infection. Leaves from each plant were sampled and assayed for CSVd as described. Healthy and CSVd-infected chrysanthemum (cv. Shamrock) were used as controls. In the second trial, 5 chrysanthemum cvs. plants derived from a clean stock program were used for mechanical inoculation studies. On 4 plants of each variety, several leaves were dusted with fine carborundum powder and rub-inoculated with a macerate of CSVd-infected Shamrock leaves in ELISA extraction buffer.

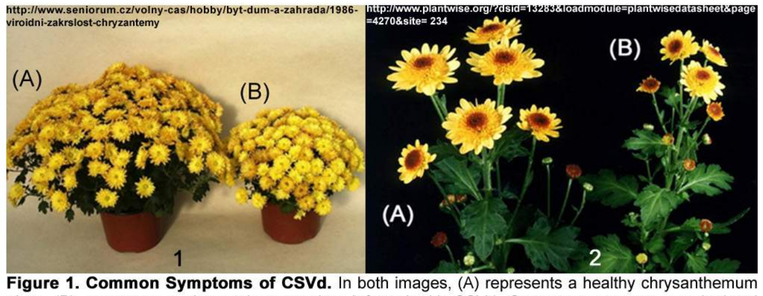


Figure 1. Common Symptoms of CSVd. In both images, (A) represents a healthy chrysanthemum plant, (B) represents a chrysanthemum plant infected with CSVd. Common symptoms are depicted above, including: Image 1[3]) Shortened stems, and image 2[4]) Uneven flowering, and reduced flower size.

Results

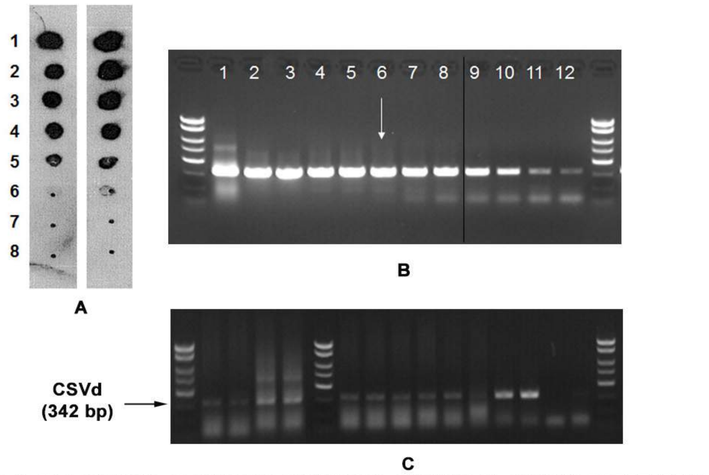
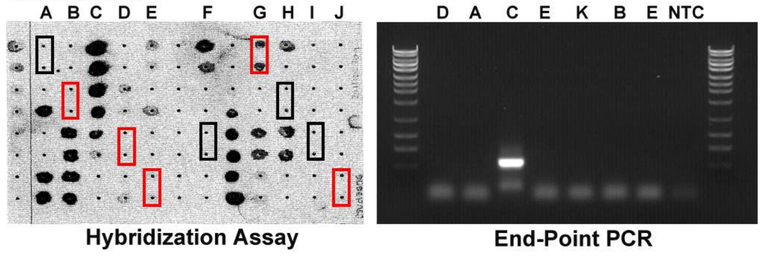


Figure 1. The sensitivity of membrane hybridization compared to one-step RT-PCR CSVd detection. A+B) Ten-fold serial dilutions of CSVd transcripts were prepared from a positive control sample with a copy number of 1.43×10^{12}. A) The membrane hybridization technique: 3µl of each dilution was loaded on the membrane. The detection limit was reached at the 6th dilution; a copy number of ~ 1.43×10^{7}. B) The one-step RT-PCR CSVd detection system: 2 µl of RNA was used for the CSVd one-step RT-PCR. 15 µl of PCR sample was loaded on a 1.5% agarose gel. One-step RT-PCR was able to detect a positive signal even from a copy number of 1.43×10^{1} (6 million times more sensitive than membrane hybridization). C) A one-step RT-PCR was performed using 10 samples that tested negative using membrane hybridization. As the membrane hybridization technique is unable to detect CSVd infections with a copy number lower than 1×10^{6}, these positive samples would have appeared negative using that method.



A	B	C	D	E	F	G	H	I	J	K
CChMVd	PSTVd	CSVd	ASBVd	TCDVd	HSVd	CEVd	CCCVd	ASSVd	TPMVd	HLVd

Figure 2. The specificity of membrane hybridization compared to one-step RT-PCR for CSVd detection. Eight different viroids were used to test cross-reactivity for both methods. The boxes indicate where other viroid samples were loaded. Their identification can be found in the table below both images. Red boxes in the hybridization assay photo indicate false positives (i.e. a positive signal was detected for a different viroid). This can be compared to the one-step RT-PCR, which has extremely specific primers to detect CSVd, and thus showed a 0% false-positive rate. Thus, one-step RT-PCR is a much more specific method for CSVd detection.

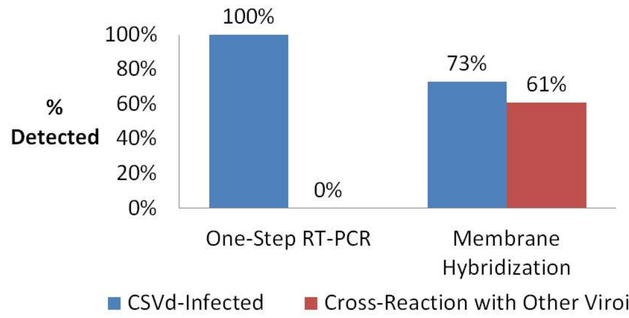


Figure 3. Summary of the sensitivity and specificity of the membrane hybridization technique compared to one-step RT-PCR for CSVd detection. False positive and false negative rates were determined by using 48 CSVd-infected plants and 9 other viroids, in duplicate. These samples were randomized, and experimenters were blinded to the identification of each sample. The one-step RT-PCR method was able to detect 48/48 (100%) of the CSVd-infected plants, yet detected none of the other viroids. On the contrary, the membrane hybridization technique only detected 35/48 (73%) of the CSVd-infected plants, while also detecting 11/18 (61%) of the other viroid samples, giving rise to a high false-positive rate.

Table 1. Comparative specifications of the membrane hybridization and the one-step RT-PCR CSVd detection methods. Not only does the membrane hybridization technique take ~20 hours, the sensitivity, specificity, PPV, NPV are all lower than one-step RT-PCR, which takes just 3 hours. The one-step RT-PCR method is extremely sensitive and specific, detecting CSVd infections in all positive samples despite the copy number, and showing no amplification when tested with related viroids.

	Membrane Hybridization	One-Step RT-PCR
Maximum Sample #	96	96
Processing Time	20 hours	3 hours
Sensitivity	73%	100%
Specificity*	39%	100%
Positive Predictive Value	76%	100%
Negative Predictive Value	35%	100%

* Based on cross-reactivity with other viroids

Conclusions

- Norgen's Viroid RNA Purification Kit isolated CSVd RNA successfully from all samples, including low copy number samples.
- Norgen's one-step RT-PCR CSVd detection system is a rapid, sensitive method for the detection of CSVd from infected chrysanthemum plants.
- The one-step RT-PCR method was found to be extremely sensitive, detecting CSVd from samples containing copy numbers as low as ~10. This compares to the membrane hybridization technique, which had a detection limit of 1×10^{7}. Based on these statistics, the one-step RT-PCR is 1×10^{6} times more sensitive than the membrane hybridization technique.
- The one-step RT-PCR method was also found to be extremely specific. While it did not display a positive result for any of the 18 samples of other viroid species, the membrane hybridization technique was found to show positive results for 11/18 (61%) of these viroid samples.

References

1. Dimock, A. W. (1947). Chrysanthemum stunt. N.Y. State flower grower's Bulletin. 26, Oct. p. 2.
2. Hollings, M., and Stone, O. M. (1973). Some properties of chrysanthemum stunt: A virus with characteristics of uncoated ribonucleic acid. Annals App Biol. 74: 333-48.
3. Konicek, J. (2008). Dwarf chrysanthemum viroid. Život90. Retrieved online on Feb. 10, 2012 from: http://www.seniorum.cz/volny-cas/hobby/byt-dum-a-zahrada/1986- viroidni-zakrslost-chryzantemy.
4. CABI. (2012). Measles of chrysanthemum (Chrysanthemum stunt viroid). Plantwise. Retrieved online on Feb. 10, 2012, from: http://www.plantwise.org/?dsid=13283&loadmodule=plantwisedatasheet&page=4270&site=234

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