

## Characterization of Three Novel Viruses Infecting Raspberry

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### Abstract

During routine graft indexing at the USDA-ARS Horticultural Crops Research Laboratory in Corvallis, Oregon, USA, a 'Glen Clova' plant that originated in Europe, induced severe symptoms on indicator plants causing mottling, epinasty and apical necrosis. Testing for all *Rubus* viruses with available laboratory based detection tests failed to identify a known virus in the plant. In an effort to determine the causal agent(s) of the symptoms, dsRNA was extracted from the 'Glen Clova' plant and cloned. Sequence analysis revealed the presence of three novel viruses in the plant, temporarily designated as Glen Clova virus -1, -2 and -3 (GC-1, -2, -3). GC-1 is a novel closterovirus with sequence similarity to *Citrus tristeza virus*, GC-2 is a member of the family *Flexiviridae* and shares greatest similarity with members of the virus family that infect rosaceous hosts. GC-3, a unique plant virus, belongs to the *Dicistroviridae*, a family of picorna-like insect viruses and is similar to a virus identified recently in strawberry. RT-PCR based detection protocols have been developed for the three viruses. GC-1 has been identified in several raspberry plants from the state of Washington. One of these plants, a yellow raspberry, did not have any other detectable viruses. When leaves from this plant were used to graft *R. occidentalis*, a standard indicator for most viruses of *Rubus*, the plant developed mosaic symptoms but not the tip necrosis and epinasty. Testing of the graft and aphid transmissible virus-like agents of *Rubus* from the UK is currently underway through a collaborative project with colleagues at SCRI in Invergowrie, Scotland. Also, transmission studies are currently underway.

### INTRODUCTION

A collection of *Rubus* and *Fragaria* cultivars that have been tested and found free of known viruses is maintained at the USDA-ARS Horticulture Crops Research Laboratory. Before new genotypes are added to this collection they are tested for viruses by grafting onto indicator plants and if a test is available, by ELISA and/or PCR. Over the past several years our laboratory has been concentrating on the development laboratory based diagnostic tests for *Rubus* viruses that currently are only detected by grafting. Efforts to efficiently clone double-stranded RNA (dsRNA) have resulted in an efficient method to obtain sequence and develop diagnostic RT-PCR tests for previously uncharacterized viruses (Tzanetakis et al., 2005). Virus-free plants very rarely have high molecular weight dsRNAs, which is produced during infection and replication of RNA viruses, either as genomic forms of some plant viruses, replication intermediates or bi-product of RNA replication (Valverde et al., 1990). Thus, the presence of such molecules in plants is an indication of virus infection. This dsRNA is quite stable and relatively easy to purify compared to single-stranded RNA from most plants including *Rubus* spp. and has been used as a starting point for the development of diagnostic tests. Known sources of virus infected *Rubus* material from North America, as well as virus sources identified from the testing of material that has been submitted for inclusion in the virus-tested collection and plants submitted because they have 'virus-like' symptoms have been assembled in our laboratory. Recently, a 'Glen Clova' plant was obtained that gave mottling, epinasty, chlorosis and apical necrosis when grafted onto the indicator *Rubus*

*occidentalis*, cv. 'Munger' (Fig. 1). This communication briefly describes the finding of three novel viruses in this 'Glen Clova' plant.

## MATERIALS AND METHODS

cDNA was acquired from dsRNA templates extracted from the 'Glen Clova' plant and cloned as described (Tzanetakis et al., 2005) without the use of restriction endonucleases. DNA sequencing was performed by MacroGen Inc. (Seoul, Korea) in a ABI3730 XL automatic DNA sequencer. Sequence identification was completed using BLAST (Altschul et al., 1997) and the consensus sequences were obtained utilizing the CAP3 software (Huang and Madan, 1999). Phylogenetic analysis was done using ClustalW (Thompson et al., 1994) with Kimura's correction and bootstrap analysis consisting of 1000 pseudoreplications.

The techniques described for *Blackberry yellow vein associated virus* detection (Susaimuthu et al., 2006) were used for the detection of GC-1, GC-2 and GC-3. For the development of detection primers generally 3 or 4 sets of primers were designed that should yield amplicons between 300 and 600 bp in size and with an annealing temperature of 55°C. After evaluations for specificity and sensitivity using a standard set of parameters for the PCR reactions a suitable set of primers is selected for detection. Primers are evaluated in this manner in order to have all tests for *Rubus* viruses that are developed use the same set of parameters for the PCR assays. Primers used for detection are shown in Table 1.

## RESULTS

When the dsRNA obtained from the 'Glen Clova' plant under investigation was separated on an agarose gel, a complex pattern was obtained suggesting that multiple viruses were present in the plant. After cloning and sequencing, three novel viruses were identified in the 'Glen Clova' plant. Partial sequence was obtained for each of the viruses and primers designed for detection by RT-PCR. Using these primers GC-1 was identified in several raspberries from Washington, a red raspberry and a yellow raspberry, both from breeding programs. When the yellow raspberry, infected only with GC-1, was grafted onto 'Munger' mild mottling symptoms were observed (Fig. 2). This suggests that the severity of the symptoms with the 'Glen Clova' may have resulted from synergistic affects of the three viruses or caused by one of the other viruses.

Several sets of detection primers were developed based on the sequence information obtained for each virus. Each set of primers was then tested against several sources of the virus and the set(s) that works well in a standard PCR protocol was selected for use (Fig. 3). In this way, all virus tests based on RT-PCR can be completed with a common set of parameters for the PCR, which allows running all PCR tests for a host plant at the same time.

In phylogenetic analysis, GC-1 clusters with the *Closterovirus* genus, which represents the aphid-transmitted members of the *Closteroviridae* family. Based on the highly conserved HSP70h and RNA dependent RNA polymerase genes in the *Closteroviridae*, GC-1 is related most closely to *Citrus tristeza virus* (Fig. 4A). The *Flexivirus* (GC-2) is most closely related to several members of the *Flexiviridae* family of plant viruses that infect Rosaceous hosts (*Cherry green ring mottle virus* and *Cherry necrotic rusty mottle virus*) (Fig. 4B). The third virus (GC-3) is related to a group of insect viruses, which was quite surprising (Fig. 4C).

## DISCUSSION

There are more than 20 viruses reported to infect *Rubus* (Converse, 1987). With the application of molecular biology to the characterization and detection of viruses of woody plants it is becoming clear that many of the virus diseases are caused by mixed infections with two or more viruses. In the example given here, the 'Glen Clova' plant would have been listed as positive for raspberry mosaic disease based on graft analysis. This is the first time that any RNA virus(es) other than Black raspberry necrosis virus has

been associated with this disease. It is yet unclear which one or combination of the three viruses causes the symptoms observed upon grafting onto 'Munger'. It is clear that the closterovirus by itself does not cause the severe symptoms observed on the grafted plants, since another raspberry plant was identified that was infected only with this virus and when grafted onto 'Munger' did not induce the severe symptoms, but rather mottling symptoms of the leaves. Since plants singly infected with the *Flexivirus* or the picorna-like virus were not identified it was not possible to infect plants with all combinations of the three viruses identified from the 'Glen Clova' plant.

Based on sequence comparisons, GC-1 is related most closely to the viruses in the genus *Closterovirus* in the family *Closteroviridae*. This is interesting since all members of this genus are aphid-transmitted as is the virus complex that causes raspberry mosaic disease (Converse, 1987). The vectors of the other two viruses identified are unknown. Vectors for members of the family *Flexiviridae* include: aphids, mites, fungi or whiteflies and for many members of this family the vectors are unknown. The picorna-like virus was somewhat surprising, but similar to a virus recently identified in strawberry (Tzanetakis et al., 2005). The role of picorna-like insect viruses in plants should not be a complete surprise. Hundreds of species of insects feed on plants and the transfer of an insect virus to plants should be expected. However, for such a virus to replicate in plants and move systemically it would have to acquire a movement protein, common to all plant viruses. This reasonably could occur via a recombination or template switching event in plants that were doubly infected with another virus. There are other well known examples of viruses that can infect plants and insects such as the Phytorhabdoviruses that infect plants and aphids, and the Tospoviruses that infect plants and thrips.

Future studies will attempt to separate out the three viruses and create mixed infections with all possible combinations to understand which virus combinations cause the severe symptoms observed. Additionally, transmission studies will be undertaken to identify vectors of the three viruses, which will be important in developing control strategies for the disease(s) caused by these viruses.

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## Tables

Table 1. List of primers used for RT-PCR detection of GC-1, -2 and -3 viruses.

GC-1 F	<i>CGAAACTTYTACGGGGAAC</i>	
GC-1 R	<i>CCTTTGAAYTCTTTAACATCGT</i>	452 bases
GC-2 F	<i>CACCTAGCAGCCTTGA</i>	
GC-2 R	<i>TGGTTTGACCAGCGAT</i>	511 bases
GC-3 F	<i>GCCATCACTAACAGGCA</i>	
GC-3 R	<i>CTCGCTGACGGGTCATA</i>	452 bases

## Figures



Fig. 1. Symptoms on *Rubus occidentalis* 'Munger' after grafting with 'Glen Clova' containing three novel viruses.



Fig. 2. Symptoms on 'Munger' grafted with yellow raspberry found to be infected with only GC-1 virus. Left: Leaf from grafted plant showing mottling symptoms; right: leaf from ungrafted 'Munger'.

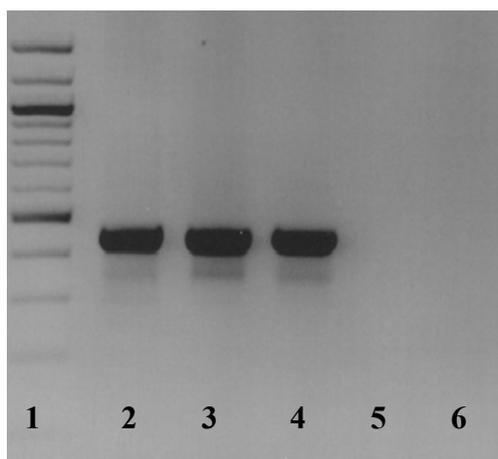


Fig. 3. Detection of GC-1 virus using primers that amplify a 452 bases fragment of the HSP70h. Lane: 1, 100 bp DNA ladder; 2, Glen Clova; 3, Red raspberry; 4, Yellow raspberry, the latter two from Washington; 5 & 6, healthy red and black raspberry, respectively.

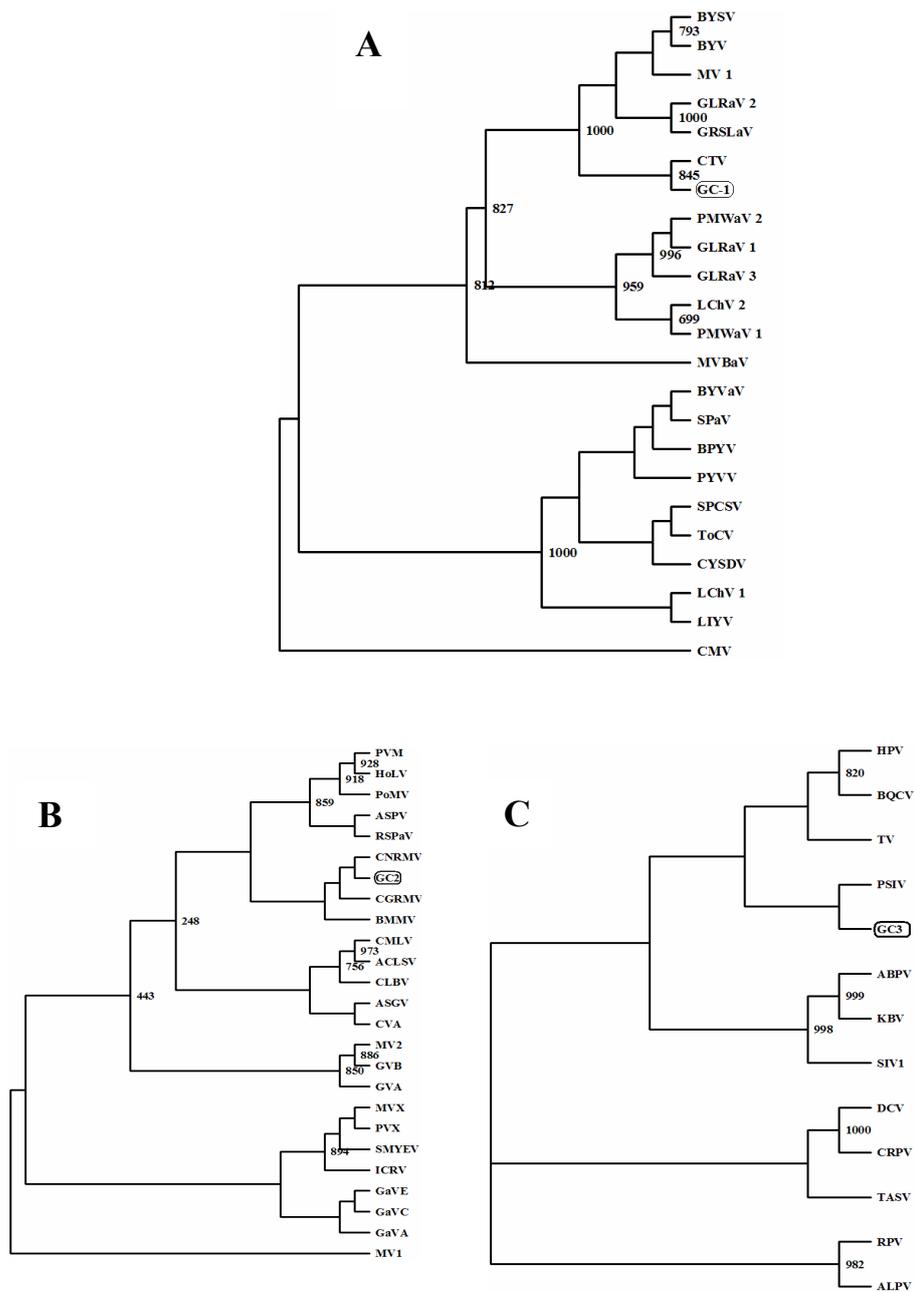


Fig. 4. Cladograms of, A, the polymerase of GC-1; B, the polymerase of the GC-2; and C, the protease of GC-3 that were cloned from 'Glen Clova'.

