Complete Nucleotide Sequence and Properties of *Raspberry Mottle Virus*

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**Abstract**

During routine graft indexing of Rubus clones at the USDA-ARS Horticultural Crops Research Laboratory in Corvallis, Oregon, a ‘Glen Clova’ plant that originated in Scotland induced severe symptoms including stunting and apical necrosis when grafted on ‘Munger’ black raspberry (*Rubus occidentalis*). Cloning of viral dsRNA obtained from the plant and sequence analysis indicated the presence of a novel virus, a member of the *Closteroviridae* and possibly additional viruses. Detection protocols developed against the closterovirus were used and identified the virus in breeding and field material from Washington State as well as several additional raspberry clones from Scotland. Graft indexing of selection ‘WSU-991’ on *R. occidentalis* induced mottling symptoms and was free of other known raspberry viruses. The virus was thus temporarily designated as *Raspberry mottle virus* (RMoV). The complete nucleotide sequence of the virus has been obtained and exceeds 17 kilobases. Phylogenetic analysis indicated that RMoV is closely related to members of the *Closterovirus* genus, the aphid-borne closteroviruses. The genome organization is an intermediate between *Citrus tristeza virus* and *Beet yellows virus* with two putative leader proteases and a conserved array of open reading frames at the 3’ proximal terminus. Currently, additional graft indexing tests are in progress to examine the possibility that RMoV is the causal agent of Raspberry leaf mottle or Raspberry leaf spot diseases previously identified in Rubus.

**INTRODUCTION**

Several virus and virus-like agents infect *Rubus* spp. (Converse, 1987). Most of those agents are asymptomatic in commercial cultivars due to the tolerance introduced in the breeding process. Losses from these latent infections cannot be estimated easily when symptoms develop in the field in plants infected with multiple viruses. In these cases, losses are substantial, and multiple infections can even cause plant death.

Since several of the agents that infect *Rubus* remain uncharacterized, grafting onto indicators is routinely used for detection of viruses. A raspberry plant ‘Glen Clova’ (GC-8) that originated at Scottish Crops Research Institute (SCRI), Scotland and was maintained in an insect-free screenhouse at the Horticultural Crops Research Laboratory (HCRL) in Corvallis, Oregon caused severe symptoms when grafted onto black raspberry ‘Munger’. The symptoms included mottling, stunting and apical necrosis, typical of raspberry mosaic disease (RMD), one of the most important viral diseases of *Rubus*. RMD was first described in the 1920s and graft-transmissible agents were associated with it in the 1950s. Four virus and graft-transmissible agents are now associated with the disease: *Black raspberry necrosis virus*, *Black Rubus yellow net virus*, *Raspberry leaf mottle virus* (RLMV) and *Raspberry leaf spot virus* (RLSV) (Converse, 1987) with the latter two being uncharacterized at the molecular level. Given the importance and the complexity of the disease, we decided to investigate the agents that were present in the GC-8 plant. Using double stranded RNA (dsRNA) as a template for shotgun cloning, we identified three novel viruses in the GC-8 plant (Martin and Tzanetakis, 2008). This communication presents data towards the characterization of one of these viruses that was named *Raspberry mottle virus* (RMoV). This novel virus was found in several plants including breeding and field material from Washington State as well as in plants that...
harbor the type isolates of RLMV, RLSV and Raspberry vein chlorosis virus (RVCV). This raises the possibility that some of these ‘type isolates’ may actually be mixed infections.

MATERIALS AND METHODS

Six raspberry plants were used as virus sources in these studies: 1) the GC-8 plant used in the majority of the experiments was obtained from SCRI; 2) a raspberry breeding accession (WSU-991) was sent to our laboratory for virus indexing by Dr. P. Moore (Puyallup, Wash.). This plant caused mottling symptoms on ‘Munger’ indicators after grafting. 3) A ‘Meeker’ grown in northern Washington (WA) exhibiting mottingling symptoms was chosen among the many plants with identical symptoms for further analysis. Dried tissue from RLMV, RLSV and RVCV-infected plants, obtained from SCRI, was assayed for the presence of RMoV. Field sources were also tested for the presence of Raspberry bushy dwarf virus, Strawberry necrotic shock virus, Black raspberry necrosis virus and Blackberry yellow vein associated virus.

For mechanical inoculations, Chenopodium quinoa, Cucumis sativus, Nicotiana benthamiana, N. occidentalis, N. tabacum and Tetragonia tetragonoides were mechanically inoculated with a homogenate of young, fully developed leaves from the ‘Glen Clova’. All herbaceous indicator plants were tested for the presence of RMoV by reverse transcription-polymerase chain reaction (RT-PCR) approximately one month after inoculation.

Double-stranded RNA purification and cloning were done as described previously (Tzanetakis et al., 2005a) from isolates, GC-8, WA and WSU-991. RNA extractions and RT-PCR amplification was essentially done as described by Halgren et al., 2007). Primers CPhF/CPhR and CPF/CPR (Table 1) were developed and used to amplify a 452 base fragment of the coat protein homolog (CPh) and a 514 base fragment of the major coat protein (CP) genes, respectively.

The genome of isolate GC-8 was acquired using the methods described by Tzanetakis et al. (2005b). ClustalW (Thompson et al., 1994) was used for phylogenetic analysis using neighbor-joining and 1000 pseudoreplicates in the bootstrap analysis.

RESULTS AND DISCUSSION

Graft-transmissible agents have been associated with raspberry mosaic disease (RMD) for more than 50 years, but only two of them are characterized at the molecular level (Jones et al., 2002; Halgren et al., 2007). The symptoms on indicators grafted with leaves of the GC-8 plant were typical of the disease; we tried to identify which of the RMD agents were present in this plant. Three new viruses were identified (Martin and Tzanetakis, 2008), indicating that RMD may be caused by previously unrecognized viruses or a complex of described and previously undescribed viruses. This communication focuses on one of the viruses, a novel member of the Closteroviridae, RMoV.

Graft indexing on R. occidentalis indicators using GC material caused severe symptoms about three weeks after grafting. Symptoms included mottling, severe stunting and apical necrosis (Fig. 1). Mottling was observed in a yellow-fruited raspberry breeding accession from Washington (WSU 991) free of the other viruses found in GC (Fig. 1).

RMoV was not found to be mechanically transmitted to the range of herbaceous indicators used in our studies. DsRNA extracted from both GC and WSU-991 had multiple bands, unlike healthy ‘Munger’ seedlings where no dsRNA was obtained (data not shown). The pattern of the dsRNA was similar to that obtained from closteroviruses (Tzanetakis et al., 2005b; Tzanetakis et al., 2005c), with a high MW band of about 17 kilobases and several lower MW bands, probably corresponding to the virus subgenomic RNAs (Fig. 2). The dsRNA was used as a template for cloning and the sequences obtained were compared with sequences found in GenBank (Altschul et al., 1997). This disclosed the presence of a virus, closely related to members of the family Closteroviridae verifying the dsRNA patterns observed.
The genome of the GC-8 isolate of RMoV is 17,481 nucleotides (nt) and encodes 10 ORFs. The first AUG is found between nt 340–342 and is the start codon of the 2819 amino acid (aa), the 313 kDa multifunctional 1a protein. The N’ terminus of protein has the two papain-like leader proteases (L1-Pro and L2-Pro, respectively) of the virus. The autocleavage of the two proteases releases a 236 kDa protein with signature methyltransferase motifs at the N-terminus and RNA helicase motifs at the C-terminus. The region between the methyltransferase and helicase contains four putative transmembrane domains (aa 1789–1812, 1824–1846, 1856–1878, 2050–2072) (Krogh et al., 2001). The virus RNA dependent RNA polymerase (RdRp, 1b) is probably expressed by a +1 ribosomal frameshift, as is the case with other members of the family (Martelli et al., 2002). The 54 kDa polymerase domain is 467 aa and has the eight conserved motifs of the RNA dependent RNA polymerases identified by Koonin (1991). There is a 645 nt intergenic region between the RdRp and a small ORF (ORF 2), which is located between nt 10846–11031 encoding a 7 kDa protein. This 7 kDa protein contains a transmembrane domain between residues 31 and 53 (Krogh et al., 2001). A similar ORF (ORF 3) is found between nt 11082–11237 encoding a 6 kDa hydrophobic protein with a transmembrane domain found between residues 11 and 33. ORF 4 (nt 11244–13043) encodes the HSP70h of the virus, the hallmark gene of all closteroviruses. The 65 kDa protein has the five conserved ATPase motifs of HSP70s between residues 3 and 336 (Bork, 1992) probably involved in virus movement. ORF 5 (nt 12943–14478) encodes a putative 58 kDa protein. Sequence analysis indicated that this protein is similar to closterovirus CPh (Napuli et al., 2003), another closterovirus movement proteins (Dolja et al., 2006). ORF 6 and 7 (nt 14555–15247 and 15372–15968) encode the two coat proteins of the virus: the 25 kDa minor coat protein and the 22 kDa CP, respectively. The proteins encapsidate the genome and are also involved in virus movement (Dolja et al., 2006). ORF 8 (nt 16026-16556) encodes a putative 20 kDa protein. BLAST searches failed to reveal any similarity of the protein with other plant virus proteins and its function remains unknown. The final ORF (nt 16628–17248) encodes a 23 kDa protein, an ortholog of an RNA silencing suppressor of BYV (Reed et al., 2003). The 3’ terminus is 233 nt and contains an adenosine rich region near the end, also found in other members of the family (Tzanetakis et al., 2005b), with unrecognized function.

Phylogenetic analysis using two regions of the closteroviral genome, the polymerase and the HSP70h, reveals clustering of RMoV with members of the Closterovirus genus as predicted from sequence analysis (Fig. 3).

RMoV primers developed against the HSP70h of isolate GC-8 failed to detect this virus in plants from northern Washington that exhibited mottling and decline. Many of these symptomatic plants were also negative for Raspberry bushy dwarf virus, Strawberry necrotic shock virus, Black raspberry necrosis virus and Blackberry yellow vein associated virus. DsRNA was extracted and cloned from a northern Washington plant (WA) and a portion of the HSP70h gene was obtained. Sequence comparison of the GC-8 and WA plants revealed that sequences belonged to the same virus. The nucleotide (nt) sequence identity of an approximate 500 base region was only 79%.

In order to study the sequence diversity observed in portions of the HSP70h and to develop optimal detection protocols, the HSP70h and the CP from the WA as well as the RLMV isolates were obtained. The HSP70h of WA is 598 aa long, in contrast to the 599aa of the GC-8 isolate. The HSP70h open reading frame (ORF) has 79% nt identity between isolates, while they share 92 and 96% aa identity and similarity respectively. The CP ORF is 198 aa long with over 97% nt and 98% aa identity between isolates. Comparison of four genomic regions of the GC-8 and WSU-991 isolates, indicating the 1300 5’ proximal bases, showed that the two isolates share 97–98% nucleotide identity, suggesting that the two isolates are very similar; thus WSU 991 was not further investigated.

Using the sequence data obtained for the GC-8, WA, WSU-991 and RLMV we optimized two sets of detection primers that were used successfully in all six isolates of RMoV of this study (GC-8, WSU-991, WA, RLM, RLS and RVC).
Field plants from northern Washington with mottling symptoms were not universally infected with RMoV. The above observations indicate that RMoV is latent in most cultivars and possibly induces symptoms when found in association with other agents.

RMoV is probably widespread, as it was identified in plants from both Europe and the United States. The diversity between the GC-8 and WA isolates is not indicative of strain distribution due to geographic isolation, as WSU-991 is closely related to GC-8 although it originated in an area proximal to that of the WA isolate. At this time it can not be ruled out that RMoV is the causal agent of one of the graft-transmittable diseases described for Rubus spp., although RMoV symptoms on grafted indicators do not correlate with those described for the graft-transmittable diseases of Rubus spp. (Converse, 1987).

**Literature Cited**


Tzanetakis, I.E., Keller, K.E. and Martin, R.R. 2005a. The use of reverse transcriptase for efficient first and second strand cDNA synthesis from single and double-stranded


**Tables**

Table 1. List of the oligonucleotide primers used for detection of Raspberry mottle virus.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5'-3')</th>
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<tr>
<td>CPhF</td>
<td>CGAAACTTTYTACGGGGAAC</td>
</tr>
<tr>
<td>CPhR</td>
<td>CCTTTGAAYTCTTTAACATCGT</td>
</tr>
<tr>
<td>CPF</td>
<td>GTAAGGAGATATGGCGGA</td>
</tr>
<tr>
<td>CPR</td>
<td>CAGTATGGCAGCCTCTTTG</td>
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**Figures**

A                           B

Fig. 1. A) Symptoms of ‘Munger’ grafted with Glen Clova tissue. Arrows point to the necrosis of the young leaves. B) Leaf symptoms of ‘Munger’ grafted with Raspberry mottle virus ‘WSU-991’ isolate. Left: Leaf from grafted plant showing mottling symptoms; right: leaf from ungrafted ‘Munger’ seedling.
Fig. 2. Double-stranded RNA (dsRNA) extracted from GC-8 infected material. Left: dsRNA obtained from RMoV infected plant, free of known Rubus viruses. Arrowheads point to a predominant 1.2 kilobases and a ~17 kilobases band; right: dsRNA pattern of Beet yellows virus used as standard.

Fig. 3. Phylogram of the (A) polymerase conserved motifs and (B) heat shock protein 70 homolog of Raspberry mottle virus (RMoV) isolate GC-8 and other closteroviruses. CMV and the Arabidopsis protein are used as outgroups for the polymerase and the heat shock protein 70 homolog, respectively. Bootstrap values are shown as percentage values and only the nodes over 70% are labeled. The bars represent 0.1 amino acid changes per site.