Identification and characterization of Raspberry mottle virus, a novel member of the \textit{Closteroviridae}

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Received 17 February 2007; received in revised form 12 March 2007; accepted 13 March 2007
Available online 19 April 2007

Abstract

Raspberry mosaic is one of the most important viral diseases of raspberry. Four virus and virus-like agents, two of which are poorly characterized, have been implicated in the disease complex based on symptom development in \textit{Rubus} indicators. Three novel viruses were identified in a red raspberry plant that caused typical raspberry mosaic symptoms when grafted onto indicators. This communication focuses on one of these viruses, Raspberry mottle virus (RMoV), a new member of the family \textit{Closteroviridae}. The complete nucleotide sequence of RMoV has been determined and exceeds 17 kilobases encoding 10 genes. The genome organization of RMoV is similar to that of \textit{Beet yellows virus}, the type member of the \textit{Closterovirus} genus, and phylogenetic analysis using the polymerase conserved motifs and the heat shock protein 70 homolog revealed a close relationship of RMoV with \textit{Strawberry chlorotic fleck associated virus} and \textit{Citrus tristeza virus}, which suggests the possibility of an aphid vector. The virus was detected in symptomatic raspberry plants in production areas in mixed infections with several other viruses, indicating that RMoV may impact raspberry production.

Keywords: \textit{Closterovirus}; \textit{Crinivirus}; \textit{Rubus}; dsRNA; Phylogenetic analysis; Detection

1. Introduction

More than 40 virus and virus-like agents infect \textit{Rubus} spp. (raspberry and blackberry) worldwide (Converse, 1987b; Halgren et al., 2007; Jones et al., 2006; Martin et al., 2004; Tzanetakis and Martin, 2004), many of which cause significant yield losses (Strik and Martin, 2003; Halgren et al., 2007). Unlike viruses causing symptoms and devastating diseases, there are several that remain latent and cause symptoms only when found in complexes with other viruses and graft-transmissible agents (Jones, 1980, 1982), making their impact on yield and fruit quality difficult to determine. Detection of viruses lacking serological or nucleic acid based methods is achieved by grafting suspect material onto indicator plants.

The USDA-ARS Horticultural Crops Research Laboratory (HCRL) in Corvallis, Oregon, maintains a collection of \textit{Rubus} accessions for breeding purposes, and graft indexing is performed routinely on these plants for identification of pathogens.

One symptomless red raspberry accession, ‘Glen Clova’ #8 (GC-8), that originated in Scotland and is maintained in an insect-free screenhouse, caused severe symptoms including leaf mottling, epinasty and apical necrosis when grafted onto black raspberry (\textit{R. occidentalis}) ‘Munger’ indicators demonstrating it was infected with one or more components of the raspberry mosaic disease (RMD). RMD was first recognized in the 1920’s and is likely caused by various combinations of four or more viruses including \textit{Black raspberry necrosis virus}, \textit{Rubus yellow net virus}, Raspberry leaf mottle virus (RLMV) and Raspberry leaf spot virus (RLSV) (Converse, 1987b), with the latter two being uncharacterized at the molecular level. Double-stranded RNA (dsRNA) was extracted, reverse transcribed, cloned and sequenced, which resulted in the identification of three novel viruses (Martin and Tzanetakis, 2005). One of the new viruses, hereafter named Raspberry mottle virus (RMoV), is presented in this communication.

While the study with isolate GC-8 was in process, unusual mottling symptoms were observed on plants in a red raspberry plantation in northern Washington. Using the techniques employed in the GC-8 virus characterization, we found that these plants were also infected with RMoV, evidence of the
distribution of the virus in both North America and Europe. This finding led to the detailed study of RMoV including the complete nucleotide sequence of the GC-8 isolate. Phylogenetic analysis showed that the virus is related to members of the genus *Closterovirus*, the aphid-borne members of the family *Closteroviridae*. The variability of the virus was studied using isolates from North America and Europe and oligonucleotide primers that can be used for detection of the virus in field grown plants were developed.

2. Materials and methods

### 2.1. Plant material

The GC-8 plant used in the majority of the experiments described was obtained from the Scottish Crops Research Institute (SCRI) as breeding material and maintained in an insect-proof greenhouse in Corvallis, Ore. A yellow raspberry breeding accession from the raspberry program in Washington (WSU-991) was sent to our laboratory for virus indexing by Dr. P. Moore (Puyallup, Wash.). This plant caused mottling symptoms on the indicator ‘Munger’ after grafting. A plant of ‘Meeker’ grown in northern Washington (WA) exhibiting mottling symptoms was chosen among the many plants with identical symptoms for further analysis. Finally, dried tissue from RLMV, RLSV and Raspberry vein chlorosis virus (RVCV)-infected plants, obtained under permit from SCRI, was assayed for the presence of the virus to test the possibility that RMoV is one of the previously described graft-transmissible agents of *Rubus* (Converse, 1987b).

### 2.2. Host range and transmission studies

Grafting was performed as described previously (Converse, 1987a). For mechanical inoculations, young, fully developed leaves from the GC-8 plant were collected and ground in phosphate buffered saline (PBS), pH 7.4, containing 2% nicotine, at wt/vol ratio of 1:20. Five plants of *Chenopodium quinoa*, *Nicotiana benthamiana*, *N. occidentalis*, *N. tabacum* and *Tetragonia tetragonioides* (*Cucumis sativus*, *Nicotiana benthamiana*, *N. occidentalis*, *N. tabacum* and *Tetragonia tetragonioides*) were mechanically inoculated after being dusted with carborundum (600 mesh) to facilitate entry of the virus into cells. All herbaceous indicator plants were tested by reverse transcription-polymerase chain reaction (RT-PCR) approximately one month after inoculation.

### 2.3. Nucleic acids isolation

Total RNA was extracted by a combination of the Halgren et al. (2007) and Rott and Jelkmann (2001) procedures. Briefly, tissue was homogenized and processed through the isopropanol precipitation step as described (Halgren et al., 2007). The precipitated RNA was resuspended in 500 µl of wash buffer and 25 µl of glass milk (Rott and Jelkmann, 2001). The glass milk was pelleted by pulse centrifugation to 6000 rpm and washed with 500 µl of wash buffer. After a brief centrifugation, glass milk was dried in a speed vac and the RNA was resuspended in 150 µl TE. Double-stranded RNA was extracted as described (Tzanetakis et al., 2005a).

### 2.4. Detection

The reverse transcription reactions was performed as described (Halgren et al., 2007), primed with either random or specific oligonucleotide primers. Primers CPhF/CPhR and CPF/CPB (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 PCR program consisted of a denaturation step of 3 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, concluding with a step of 10 min at 72 °C, using Taq polymerase (GenScript, NJ) according to manufacturer’s instructions, and primers at 1 or 0.5 µM each (CPB and CP, respectively).

### 2.5. Cloning, sequencing and genome analysis

The complete nucleotide sequence of RMoV was obtained from the GC-8 accession and the methodology used was essentially identical to that used for another closterovirus, Mint

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**Table 1**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5′–3′)</th>
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<td>Detection</td>
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<tr>
<td>CPhF</td>
<td>CGAAACTTYTACGGGGAAC</td>
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<tr>
<td>CPhR</td>
<td>CCTTGAAATCCCTTAACATCGT</td>
</tr>
<tr>
<td>CPF</td>
<td>GTAAGGAGATATGCCCGGA</td>
</tr>
<tr>
<td>CPR</td>
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</tr>
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</table>

**Genomic variability**

| HSPA | CTCTACTAAGCAGGATCTG          |
| HSPB | CATAGTACCTTGAGGAGGC          |
| CPA  | ACACCGGAAGCTTCATTGGTTT       |
| CPB  | AATACACCTAAACGGGAGGC         |

**Genome amplification**

<table>
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</tr>
<tr>
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<tr>
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<tr>
<td>Pol 2500 R</td>
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<tr>
<td>Pol beg F</td>
<td>AGTGGTCTGATTTGGGCGCC</td>
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<td>Pol mid R</td>
<td>GAGACGAGCCGCAAGACGAGATCGAAG</td>
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<tr>
<td>Pol mid F</td>
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<td>Pol end R</td>
<td>CGAGGAAATATAGGTTGGAGCGAC</td>
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<td>Pol end F</td>
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<td>Mid F</td>
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<td>End F</td>
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virus-1 (Tzanetakis et al., 2005a; Tzanetakis et al., 2005c). RMoV-specific sequences were identified using blastn and blastx to compare with sequences found in the GenBank databases (Altschul et al., 1997). Oligonucleotide primers were developed from the RMoV sequences and used for RT-PCR amplification of the genome (Table 1). The 5′ and 3′ termini of the genome were acquired by 5′ and 3′ RACE using primers AAP and AP (Table 1; Invitrogen) as described previously (Tzanetakis and Martin, 2004). Consensus of the complete genome and all other sequences presented were acquired utilizing CAP3 (Huang and Madan, 1999) and were built using the clones from shotgun cloning where applicable, at least four clones of the RT-PCR amplicons and direct sequencing of the RT-PCR products, for an at least 5× genome coverage. The genome of RMoV has been deposited in Genbank and given the accession number DQ357218. The genetic diversity of the virus was examined by sequencing the heat shock protein 70 homolog (HSP70h; accession numbers DQ016611–EF114208) and CP genes (accession numbers DQ016612–EF114209) of the WA and RLM isolates, respectively.

The open reading frames (ORF) were identified using the ORF finder software at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Conserved protein domains were identified with CDD (Marchler-Bauer et al., 2003) and the exact amino acid (aa) regions were recognized after alignment of known closteroviral motifs using ClustalW (Thompson et al., 1994). ClustalW was also applied for phylogenetic analysis (neighbor-joining algorithm, Kimura’s correction and bootstrap analysis consisting of 1000 pseudoreplicates). Trees were viewed on TreeView (Page, 1996). The putative transmembrane domains of the proteins were predicted with TMHMM (Krogh et al., 2001) while RNA secondary structure was predicted using Mfold (Zuker, 2003).

3. Results

Severe symptoms developed on ‘Munger’ black raspberry indicators about 3 weeks after grafting with GC-8 leaves. Symptoms included mottling, severe stunting and apical necrosis (Fig. 1A). Mottling developed on ‘Munger’ after grafting with WSU-991, free of the other viruses found in GC-8 (Fig. 1B) and for this reason we propose the name Raspberry mottle for the new virus. RMoV was not mechanically transmitted to the range of herbaceous indicators used. Multiple dsRNA bands were obtained from GC-8 and WSU-991, 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, with a prominent 1.2 kilobase band, probably corresponding to virus subgenomic or defective RNAs (Fig. 2). The dsRNA was used as a template for cloning, and about 11 kilobases of the RMoV genome were obtained using the shotgun approach as determined by comparison to closterovirus sequences found in GenBank (Altschul et al., 1997).

The complete nucleotide sequence of the GC-8 isolate of RMoV has been determined and consists of 17,481 nucleotides (nt) (G + C content: 47.5%) encoding 10 putative ORFs (Fig. 3). The first AUG codon of the GC-8 isolate is found between nt 340–342. The 1a polyprotein is 2819 aa and approximately 313 kDa.

Fig. 1. (A) Symptoms of ‘Munger’ indicators grafted with ‘Glen Clova’ 8 tissue. Arrows point to the necrosis of the apical meristem. (B) Leaf symptoms of ‘Munger’ indicators grafted with the Raspberry mottle virus ‘WSU 991’ isolate. Left: Leaf from grafted plant showing mottling symptoms; right: leaf from ‘Munger’ seedling.
Fig. 2. Double-stranded RNA (dsRNA) extracted from Raspberry mottle virus (RMoV) infected material. Left: dsRNA obtained from of 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.

Both domains show greater similarity to orthologous domains of SCFaV with 52 and 58% aa identity for the methyltransferase and helicase domains, respectively. 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) although the surrounding region does not have any slippery sequences nor structures that could facilitate the frameshift (Zuker, 2003). It is possible that the frameshift is facilitated by a newly identified antisense mechanism (Henderson et al., 2006). The 54 kDa polymerase domain is 467 aa and has the eight conserved motifs of the RNA dependent RNA polymerases identified by Koonin (1991) between residues 108 and 346. The region shows the highest aa identity with orthologous domains of other members of the *Closterovirus* genus, exceeding 67% identity with all sequenced members of the group and reaching almost 80% for some isolates of CTV. 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. A Cys in position 3 indicates that the peptide probably has a similar function to the small hydrophobic peptide of *Beet yellows virus* (BYV). BYV p6 is one of the five proteins involved in virus movement. The Cys at position 3 is involved in disulfide bridge formation and dimerization of the protein, a property essential in virus movement (Peremyslov et al., 2004). ORF 4 (nt 12144–13043) encodes the HSP70h of the virus, the hallmark gene of all closteroviruses. The 65 kDa protein is involved in virus movement and virion assembly (Dolja et al., 2006) and has the five conserved ATPase motifs of HSP70s between residues 3 and 336 (Bork et al., 1992) probably involved in virus movement.

Fig. 3. Schematic representation of the genome organization of Raspberry mottle virus. Abbreviations: L1-Pro: leader papain-like protease 1; L2-Pro: leader papain-like protease 2; MT: methyltransferase; Hel: helicase; Pol: RNA-dependent RNA polymerase; HSP70: heat shock protein 70 homolog; CPh: coat protein homolog; CPm: minor coat protein; CP: major coat protein. Dashed bars represent regions of the virus acquired by shotgun cloning of double-stranded RNA. Solid bars represent the reverse transcription-polymerase chain reaction products used to acquire the genome of the virus. Primers are listed in table 1. Open reading frames are not to scale.
Fig. 4. (A) Phylogram of the polymerase conserved motifs of Raspberry mottle virus (RMoV) and other closteroviruses. Abbreviations and GenBank accession numbers: BPYV, Beet pseudo yellows virus, NP940796; BYV, Beet yellows virus, NP733949; BYSV, Beet yellow stunt virus, AAC55659; BYVaV, Blackberry yellow vein associated virus, AAV40963; CTV, Citrus tristeza virus, NP733947; CMV, Cucumber mosaic virus, NP049324; CYSDV, Cucurbit yellow stunting disorder virus, AAM73639; GLRaV 1, Grapevine leafroll associated virus 1, AAF22738; GLRaV 2, Grapevine leafroll associated virus 2, AAC40856; GLRaV 3, Grapevine leafroll associated virus 3, AAC40705; LIYV, Lettuce infectious yellows virus, AAA61798; Little cherry virus 1, NP733945; LChV 2, Little cherry virus 2, AAP87784; MVBaV, Mint vein banding associated virus, AAS57939; MV 1, Mint virus 1, AAW32893; PMWaV 1, Pineapple mealybug wilt associated virus 1, AAL66709; PMWaV 2, Pineapple mealybug wilt associated virus 2, AAG13939; PYVV, Potato yellow vein virus, CAD89680; RMoV, Raspberry mottle virus, DQ357218; SCFaV, Strawberry chlorotic fleck associated virus, DQ860839; SPaV, Strawberry pallidosis associated virus, AY488137; SPCSV, Sweet potato chlorotic stunt virus, NP733939; ToCV, Tomato chlorosis virus, AAY21795. CMV is used as the outgroup. (B) Phylogram of heat shock protein 70 homolog of RMoV and other closteroviruses. GenBank accession numbers: Arabidopsis, Arabidopsis thaliana putative heat shock protein 70, AAN71949; BPYV, AAO79386; BYSV, AAC55662; BYVaV, NP041872; BYVaV, AA490663; CTV, NP402864; CYSDV, NP51572; GLRaV 1, AAF22740; GLRaV 2, AAR21242; GLRaV 3, NP813799; LIYV, NP619695; LChV 1, NP645004; LChV 2, AF531505; MVBaV, AAS57941; MV 1, AAW32895; PMWaV 1, AAL66711; PMWaV 2, AAG13941; PYVV, CAD89682; RMoV, DQ357218; SCFaV, DQ860839, SPaV, AA092347; SPCSV, NP689401; ToCV, AF024630. The Arabidopsis protein is used as the outgroup. Bootstrap values are shown as percentage value and only the nodes over 70% are labeled. The bars represent 0.1 amino acid changes per site.

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. 4).

A primer set developed against the HSP70h of GC-8 isolate failed to detect the virus in any of WA plants that exhibited leaf mottling. These plants were tested for Raspberry bushy dwarf virus, Black raspberry necrosis virus, Strawberry necrotic shock virus and Blackberry yellow vein associated virus (BYVaV) but none of these viruses was consistently found in these plants indicating the symptoms were caused by a combination of viruses or a novel agent. DsRNA was extracted and cloned as described for GC-8 from a WA plant that exhibited leaf mottling and a portion of the HSP70h gene was obtained. The nt sequence identity of an approximate 500 base region of the gene from the GC-8 and WA plants was only 79%, while the aa identity exceeded 90% indicating that these plants were infected with RMoV.

In order to study the sequence diversity observed in portions of the HSP70h and develop optimal detection protocols, the HSP70h and the CP from the WA and the RLM isolates were obtained. The WA HSP70h is 598 aa, in contrast to the 599 aa of the GC-8 isolate. The open reading frame (ORF) has 79% nt identity between isolates, while they share 92 and 96% aa sequence identity and similarity, respectively. The CP ORF is 198 aa with over 97% nt and 98% aa sequence identity between isolates. In contrast the two genes of RLM show great similar-
ity to the GC-8 isolate with 97% nt sequence identity for the HSP70h and 96% for the CP. The aa sequences are 97 and 99% identical to the orthologous genes of GC-8. Shotgun cloning from dsRNA obtained from the WSU-991 isolate showed that the two isolates share 97–98% nucleotide identity in regions of 1a, the HSP70h and the CP (Tzanetakis, unpublished data), suggesting that the two isolates are very similar.

Because of the problems with detection of the WA isolate, several sets of oligonucleotide primers were tested for detection of RMoV. The sets presented were the two that detected all six isolates (GC-8, WSU-991, WA, RLM, RLS and RVC) in addition to isolates from black and red raspberry from the Pacific Northwest (data not shown). Validation of the tests was done by sequencing at least five of the amplicons from each primer set. They were all determined to be RMoV-specific.

More than 20 plants from northern Washington with symptoms similar to that of the WA isolate were assayed for the presence of RMoV with both detection primer sets. While the majority were found infected with RMoV, some were not found infected with the virus.

4. Discussion

Graft-transmissible agents have been associated with 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). Grafting of GC-8 onto indicators caused symptoms typical of RMD and this prompted us to identify which of the RMD agents were infecting this plant. Three new viruses were identified (Martin and Tzanetakis, 2005), indicating that RMD may be caused by previously unrecognized viruses or a complex of the new and the old viruses. This communication focuses on one of the viruses, a novel member of the Closteroviridae named RMoV.

The close relationship of the virus with members of the Closterovirus genus, the only members of the family that are potentially mechanically transmitted (Martelli et al., 2002), led to the investigation of that property. RMoV was not found to be mechanically transmitted to the range of herbaceous indicators used. This could be a property of the virus or a combination of the inefficient mechanical transmission of most closteroviruses with the inhibitors found in Rubus and related species (Fulton, 1966).

The low titer of closteroviruses (Liu et al., 2000; El Beaino et al., 2005; Tzanetakis et al., 2005c) makes detection a challenging undertaking. Because of that property, RT-PCR is the preferred detection method for closteroviruses. The disadvantage of the method is the potential of false negatives because of mismatches between primers and template that can occur in isolates as diverse as those found with RMoV. In order to minimize that possibility, two sets of primers for two genomic regions of the virus were developed for routine detection (Fig. 5).

The diversity of closteroviruses is well established (Karasev, 2000; D’Urso et al., 2003). The extreme nt diversity of the HSP70h ORF for isolates of the same species, has also been found in other closteroviruses (Tairo et al., 2005; Ruiz-Ruiz et al., 2006). The contrast between the diverse HSP70h ORF (79% nt identity) to the very similar CP ORF (97% nt identity) is a feature found in other closteroviruses and especially CTV, where sequence diversity between isolates decreases from the 5′ to 3′ end of the genome (Mawassi et al., 1996). The different size of the HSP70h is unusual, although there are reports of ORFs of considerably different sizes in closteroviruses (Tzanetakis and Martin, 2004). The consensus sequence of the terminal region of RMoV HSP70h was obtained after alignment of six clones of the PCR products, all of which were identical.

An interesting feature of the GC-8 isolate was observed when trying to obtain the 5′ terminal sequence using dsRNA as template. In the first 300 nucleotides of the genome there were two gaps that resulted in about 70 nt missing compared to the homologous region of the WSU-991 and the RLM isolates (accession numbers EF421975-EF421976, respectively).

This region of the GC-8 isolate was sequenced at least 15 times, from clones of PCR amplicons derived from three individual RT and four PCR reactions. In all cases the gaps were present. The region is not highly structured (Zuker, 2003), nor GC rich (G+C content: 47.5%). We used methyl mercury hydroxide in the denaturation step of the dsRNA and the RT was performed at

Fig. 5. Detection of Raspberry mottle virus (RMoV) using reverse transcription-polymerase chain reaction. (A) Amplification of a 452 base region of the coat protein homolog gene. Lane 1: 100 base pair marker; lanes 2–4: RMoV infected red raspberry from Scotland, red raspberry from Washington, and yellow raspberry from Washington, respectively; lane 5–6: healthy red and black raspberry seedlings. (B) Amplification of a 514 base region of the major coat protein gene. Lane 1: 100 base pair marker; lanes 2–4: RMoV infected red raspberry from Scotland, red raspberry from Washington, and yellow raspberry from Washington, respectively; lane 5–6: healthy red and black raspberry seedlings.
60 °C, minimizing the possibility of the gap regions being inaccessible during the RT reaction. With the gaps the first AUG of the genome was at position 1314. A 5′ UTR of that size in unprecedented and would give no obvious advantage to the virus. We tried to obtain the region using single-stranded RNA as template and the gaps in the sequence were absent placing the start codon of 1a at position 340–342. This finding is perplexing, but highlights the importance of closely examining anomalies observed in sequences of new viruses to ensure they are not the result of difficulties with template.

The presence of two small hydrophobic proteins in the genome of RMoV and the area of about 650 nt between the polymerase and p7 may be the remains of a larger gene that was found in that position that was abolished recently or gave rise to p7. This hypothesis is reinforced by the fact that the region lacks a start codon and has a single stop codon in one of the reading frames. Che et al. (2003) found that a class of defective RNAs of CTV are similar to the genomic RNAs of criniviruses. All sequenced members of the Crinivirus genus have a small hydrophobic protein at the end of RNA 1 and beginning of RNA 2 with the exception of BYaV and BYMV that has the two small hydrophobic proteins at the beginning of RNA 2 (Tzanetakis et al., 2006) and Lettuce infectious yellows virus that lacks the first protein (Klaassen et al., 1995). The data presented here together with the findings on the defective RNAs of CTV (Che et al., 2003), and a monopartite clustrovirus (Mint vein banding associated virus, Tzanetakis et al., 2005b) that shares similarities with all genera of the family, strengthens the argument that criniviruses evolved from a monopartite virus similar to members of the Clustrovirus genus.

The northern Washington plants with mottling symptoms were not universally infected with RMoV, which suggests that RMoV is not the sole causal agent of the symptomatology but it 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, since WSU-991 was closely related to GC-8 even though it originated in an area proximal to that of the WA isolate. The variability may be the result of genetic bottlenecks caused by vector transmissions or host passages, similar to what has been observed for CTV (Ayllon et al., 2006; Sentandreu et al., 2006).

At this time it can not be ruled out that RMoV is the causal agent of one of the graft-transmissible diseases described for Rubus spp., although RMoV symptoms on grafted indicators do not correlate with those described for the graft-transmissible diseases of Rubus spp. (Converse, 1987b). The plants that are infected with the reference isolates of RLMV, RLSV and RVCV from SCRi were infected with RMoV, making even more complex the correlation of symptoms observed in these plants and the agents present. Our observations suggest that several of the symptoms described for many of the small fruit viruses and attributed to severe strains of known viruses probably result from mixed infections of known and unknown viruses. RMoV may be part of a complex of viruses that cause the symptoms described and associated with one or several of the Rubus graft-transmissible diseases.

Acknowledgments

The authors would like to thank Ruth Price for optimizing the RNA extraction protocol. We also thank Alexander Karasev and Valerian Dolja for helpful discussions and Hugh Barker and Stuart McFarland for providing us with the SCRi Raspberry leaf mottle, Raspberry leaf spot and Raspberry vein chlorosis tissue. The project was funded by the United States Department of Agriculture.

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