A new member of the family *Reoviridae* may contribute to severe crumbly fruit in red raspberry, *Rubus idaeus* 'Meeker'

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Abstract

A virus induced crumbly fruit disease of considerable importance in 'Meeker' and other cultivars of red raspberry has been observed in northern Washington, USA, and British Columbia, Canada and to a lesser extent in the Willamette Valley of Oregon. Raspberry bushy dwarf virus (RBDV), a pollen-borne virus, has been considered the causal agent of the disease. However, dsRNA extractions from raspberry plants exhibiting severe crumbly fruit in northern Washington revealed multiple bands in addition to those corresponding to RBDV (5.5kb and 2.2kb). Sequence analyses of these dsRNAs showed the presence of two additional viruses. One has significant amino acid identity to proteins encoded by Rice ragged stunt virus (RRSV), a ten-segmented dsRNA *Oryzavirus* that belongs to the family *Reoviridae*. Thus far, all dsRNA segments, except the one that corresponds to S6 of RRSV, have been fully sequenced and found to have characteristic features of other plant reoviruses genomes. In addition, Raspberry leaf mottle virus (RLMV), a recently characterized member of the *Closteroviridae*, has also been identified from raspberries with severe crumbly fruit. These findings along with the lack of severe crumbly fruit symptoms in 'Meeker' red raspberry singly infected with RBDV in Oregon, suggest the existence of a novel virus complex associated with severe crumbly fruit in red raspberries. The complex may involve RBDV, RLMV and/or this new reovirus, provisionally named Raspberry latent virus (RpLV).

Keywords: Raspberry crumbly fruit, Raspberry bushy dwarf virus, Raspberry leaf mottle virus, Raspberry leaf spot virus, plant reoviruses.

Introduction

There are more than 40 viruses known to infect *Rubus* spp. a number of which have been described based on symptom development, mode of transmission and particle morphology (Converse, 1987). Others are less well characterized and based on symptoms caused on indicator plants. One of the viruses in this latter group, Raspberry leaf spot virus (RLSV), has been reported as a component of Raspberry mosaic disease virus complex in Europe but not in North America (Jones, 1982). Raspberry plants that tested negative for RBDV but exhibiting leaf mottling symptoms were observed in production areas in Washington, USA and British Columbia, Canada. Leaf tissue from these symptomatic plants was used for dsRNA extractions and cloning. Partial sequence information obtained from cloning revealed the presence of a new plant reovirus. Interestingly, primers made to detect the virus failed to detect RLSV in symptomatic plants in Europe, suggesting that the symptoms observed in plants in North America were caused by a different virus (Martin and Jelkmann, personal communication).

The above mentioned primers have been used as part of the routine virus testing program in raspberry plants in the USA, to detect the virus that, mistakenly, has been referred to as RLSV. Hereafter, we will refer to this new virus as Raspbery latent virus (RpLV), since it does not cause symptoms in several indicators and cultivars of Rubus in single infections. Note that the RLSV in Europe has been shown to be a variant of Raspberry leaf mottle virus (RLMV) (Tzanetakis et al., 2007) and thus, due to precedence in original description, RLMV should be the name used for these viruses (See MacFarlane – these proceedings). Interestingly, raspberry plants showing severe crumbly fruit symptoms from different fields in northern Washington have tested positive for RpLV and RLMV as well as for RBDV. Raspberry bushy dwarf virus (RBDV), a pollen-borne *Ideovirus*, has been associated with crumbly fruit disease in most red raspberry cultivars worldwide (Daubeny et al., 1982). With the finding that severely crumbly fruited 'Meeker' plants are infected with multiple viruses, the role of these additional viruses in crumbly fruit should be investigated in other cultivars and other production areas. These findings, along with the lack of severe crumbly fruit in 'Meeker' red raspberry singly infected with RBDV in Oregon, suggest a possible new virus complex associated with severe crumbly fruit in the susceptible raspberry cultivar 'Meeker' and possibly other cultivars. The complete characterization of RpLV is necessary to elucidate possible interactions with other common raspberry viruses and its implications in raspberry diseases. This communication reports the partial characterization of RpLV, including dsRNA extractions, sequence analyses, grafting onto indicators, and possible vectors.

Materials and methods

Virus source: Root cuttings from symptomatic 'Meeker' raspberry plants were obtained from production fields in northern Washington. The roots were planted, grown in one gallon pots and maintained in a greenhouse under standard conditions.

Double-stranded RNA extraction: Twenty grams of fresh leaf tissue was powdered in liquid nitrogen. DsRNA was extracted using phenol/STE buffer, and recovered by cellulose CF-11 chromatography, as previously described (Morris and Dodds, 1979). The dsRNA was treated with ribonuclease T1 from Aspergillus oryzae (Sigma) and DNAse 1 from bovine pancreas (Sigma) to remove single-stranded RNA and dsDNA, respectively. After the digestions the dsRNAs were again purified on CF-11 cellulose columns and precipitated with EtOH. The dsRNA was pelleted, dried and separated by electrophoresis through 1% agarose gels stained with ethidium bromide and visualized under UV light. All bands except the ones corresponding to RBDV (5.5kb and 2.2kb) and RLMV (17kb and 1.2kb) were gel extracted using a DNA gel extraction kit (Genescript).

cDNA synthesis, cloning and sequencing: The reverse transcription (RT) reaction was primed with the universal random primer 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3' (Froussard, 1992) and the methodology described by Tzanetakis et al., (2005) was used. Briefly, a mixture containing the purified dsRNA and primers was denatured with CH_3HgOH at room temperature for 30 min. Then, a second mix, containing reverse transcription buffer, DTT, dNTPs, and Superscript III RT (Invitrogen) was added to the denaturant mix and incubated for 50 min at 50C. The reaction was terminated by heating at 75C for 10 min. The RNA template was digested with RNAse H (Invitrogen), and then a PCR reaction containing the anchor primer 5'-GCCGGAGCTCTGCAGAATTC-3'was carried out to amplify the cDNA products. The PCR products were cloned into the TOPO TA vector (Invitrogen) and sequenced.

Genome assembly: The sequences were assembled into contigs using the assembly program CAP3, the contigs were then compared to the database at NCBI GenBank using BLAST, which returned Rice ragged stunt virus (RRSV) as the nearest relative. Contigs were aligned with RRSV and then specific primers were made to fill in the gaps. The 5' and 3' termini were obtained by poly (A) tailing of the 3' ends of dsRNAs, as described (Isogai et al., 1998), followed by RT-PCR using primers developed to known sequence near the ends and oligo dT. To confirm the last base, a 3' blocked DNA oligonucleotide was ligated to both 3' ends of the dsRNAs followed by RT-PCR using its complementary primer and specific primers for each end.

Grafting assays: Plants with single infections of RpLV or mixed infections (RLMV and RpLV) were grafted onto the *Rubus* virus indicators 'Norfolk Giant', 'Malling Landmark' and 'Munger'. Symptom development was monitored for nine weeks.

Aphid transmission assays: The large raspberry aphid Amphorophora agathonica was tested for its ability to transmit RpLV. This aphid is common in the main raspberry production areas in northern Washington where severe crumbly fruit and RpLV are very common. Also, this aphid and RpLV are much less common in the Willamette Valley of Oregon. Nonviruliferous *A. agathonica* were placed on an RpLV-infected 'Meeker' raspberry plant and allowed to feed for two weeks. Then, approximately 60 aphids were transferred to a virus-tested 'Meeker' plant and allowed a 1.5 h inoculation access period. Then the aphids were transferred serially to four additional healthy 'Meeker' plants and allowed inoculation access times of 2.5 h, 8 h, 12 h, and 24 h, respectively. After each inoculation access feeding period, six aphids were collected and tested for the presence of RpLV by RT-PCR. The aphid transmission test plants were tested for RpLV 6, 10 and 14 weeks post-inoculation by RT-PCR.

Results

Double-stranded RNA (dsRNA): In addition to the bands corresponding to RBDV (5.5kb and 2.2kb) and RLMV (17kb and 1.2kb) multiple bands were observed from crumbly-fruited raspberry plants when separated by electrophoresis on agarose gels. These additional bands migrated between ~4kb and ~1100bp of the dsDNA marker (Figure 1).

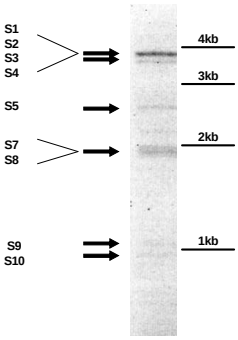

Fig. 1 DsRNA from symptomatic 'Meeker' plants separated by electrophoresis (1% agarose gel).

Sequencing: Sequencing analyses showed significant amino acid identity to different proteins encoded by Rice ragged stunt virus (RRSV). RRSV is a ten-segmented dsRNA Oryzavirus that belongs to the family Reoviridae. Terminal sequences revealed that RpLV has the conserved sequences AGUU/A and GAAUAC at the 5' and 3' termini of each RNA segment, respectively (Table 1).

Tab. 1 Conserved terminal sequences of RpLV

Genome segment	5' terminus	3' terminus
S1	**AGU**UUUA	**GCGAAUAC**
S2	**AGU**UUAU	**GGGAAUAC**
S3	**AGU**GAAA	**GCGAAUAC**
S4	**AGU**UUUA	**GCGAAUAC**
S5	**AGU**UUUU	**GUGAAUAC**
S7	**AGU**AAAA	**GCGAUUAC**
S8	**AGU**UUUA	**GCGAAUAC**
S9	**AGU**UAAA	**GUGAAUAC**
S10	**AGU**UAAA	**GCGAAUAC**

In addition, each segment has an inverted repeat that consists of 6 to 8 nucleotides adjacent to the conserved region (Figure 2). Thus far, nine dsRNA segments have been completely sequenced; the putative proteins encoded by each segment are presented in table 2. The relatedness of RpLV to RRSV and other plant reoviruses is underway and completion of the elucidation of the tenth genomic segment if it exists.

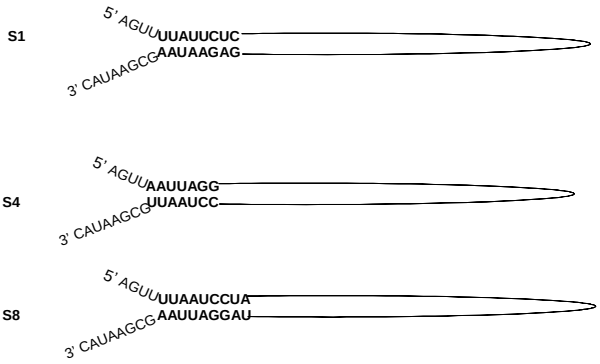

Fig. 2 Inverted repeats of genome segments S1, S4, and S8 of RpLV. The positive sense strand is illustrated. Regions adjacent to the conserved terminals are complementary.

Tab. 2 Putative proteins encoded by each genomic segment of RpLV based on amino acid identity to RRSV

RRSV Genome seg (bp)	Protein encoded by segment	Raspberry latent virus genome (bp)	Amino acid score/identity (bits/%)
1 (3849)	Spike	3948	299/35
2 (3808)	Structural	3650	251/27
3 (3699)	Structural	3566	229/29
4 (3823)	RdRp	3818	323/39
5 (2682)	Capping enzyme	2563	80/27
6 (2157)	RNA-binding	not detected	unknown
7 (1938)	Non structural	1936	79/26
8 (1814)	Autocatalytic enz	1997	30/28
9 (1132)	Spike	1141	62/24
10 (1162)	Unknown	1205	58/23

Grafting: Grafting assays showed that indicators grafted with leaves from plants singly-infected with RpLV did not exhibit symptoms; whereas plants containing mix infections with RLMV and RpLV developed symptoms when grafted onto the raspberry virus indicator 'Malling Landmark' (Figure 3), but was symptomless in 'Norfolk Giant'.

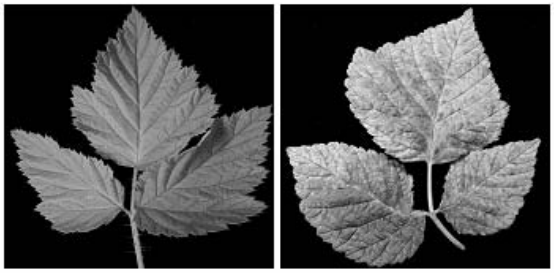

Fig. 3 Indicator raspberry 'Malling Landmark' 6 weeks post graft-inoculation. Left: Leaf grafted with RpLV only. Right: Leaf from plants grafted with RpLV and RLMV

Aphid transmission assays: RpLV was detected by RT-PCR in the fourth plant in the serial transmission tests, which had an inoculation access time of twelve hours. The virus was not detected after access feeds of 1.5, 2.5 and 8 hours and nor was there any transmission after 12 hours. Interestingly, the aphids tested positive for RpLV by RT-PCR throughout the transmission experiment. These experiments are being repeated as well as transmission tests with leafhoppers.

Discussion

The initial dsRNA extractions and limited sequence of RpLV was obtained from raspberry plants as early as 1988. However, recent observations of plants affected with severe crumbly fruit and the presence of this virus along with RLMV suggested that a virus complex may be responsible for the disease (Martin, personal communication).

Grafting assays suggest a possible synergistic effect of these two viruses when found in mix infections. It is believed, that RpLV in conjunction with RLMV and RBDV may cause severe crumbly fruit in 'Meeker'. This hypothesis will be studied by real-time PCR, fruit quality and yield evaluations of plants with single or mix infections of all combinations of the three viruses, RpLV, RLMV and RBDV.

Plant reoviruses possess terminal sequences, which are conserved at the genus level, and segment-specific inverted repeats adjacent to the conserved termini (Kudo et al., 1991; Yan et al., 1992). Sequencing results of nine dsRNA segments obtained from symptomatic raspberry plants, show those characteristics. The first three nucleotides at the 5' end (AGU) are similar to those of other reoviruses. However, the 3' terminal of RpLV seems to be unique, which indicates that the virus probably belongs to a new genus of the plant *Reoviridae*. Most viruses belonging to the family *Reoviridae* contain 10-12 dsRNA genome segments (Mertens et al., 2005). Recently, a new reovirus isolated from the mosquito *Aedes pseudoscutellaris* was found to have a genome comprised of 9 dsRNA segments (Attoui et al., 2005). Our sequence results indicate that RpLV may also have nine genome segments, although this is not definitive. One additional experiment with 'Deep Sequencing' will be performed in an effort to identify a tenth genomic segment for RpLV.

The high aphid populations in northern Washington, where severe crumbly fruit is more prevalent, prompted us to conduct transmission assays with the common raspberry aphid *Amphorophora agathonica*, despite the fact that all known plant reoviruses are transmitted by various species of leafhoppers. The result obtained from this preliminary experiment is quite interesting. If reproducible, it would indicate that the virus is transmitted by aphids in a semi-persistent manner since first several and the last serial transfers were negative. An alternative explanation could be that A. agathonica is an inefficient vector of the RpLV. Experiments with leafhoppers as vectors are underway, as well as repeating the aphid transmission tests.

At this time, the role of RpLV in severe crumbly fruit in red raspberry is unclear. Plants singly infected with RBDV, RLMV, and RpLV, are being used to create mix infections in all possible combinations. These plants will be planted in field experiments to study the impact of various virus combinations on fruit quality and yield, and also to study the interaction between viruses using real time PCR.

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