Newly identified RNAs of raspberry leaf blotch virus encoding a related group of proteins

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Members of the genus *Emaravirus*, including *Raspberry leaf blotch virus* (*RLBV*), are enveloped plant viruses with segmented genomes of negative-strand RNA, although the complete genome complement for any of these viruses is not yet clear. Currently, wheat mosaic virus has the largest emaravirus genome comprising eight RNAs. Previously, we identified five genomic RNAs for *RLBV*; here, we identify a further three RNAs (RNA6–8). RNA6–8 encode proteins that have clear homologies to one another, but not to any other emaravirus proteins. The proteins self-interacted in yeast two-hybrid and bimolecular fluorescence complementation (BiFC) experiments, and the P8 protein interacted with the virus nucleocapsid protein (P3) using BiFC. Expression of two of the proteins (P6 and P7) using potato virus X led to an increase in virus titre and symptom severity, suggesting that these proteins may play a role in *RLBV* pathogenicity; however, using two different tests, RNA silencing suppression activity was not detected for any of the *RLBV* proteins encoded by RNA2–8.

INTRODUCTION

*Raspberry leaf blotch virus* (*RLBV*) is a recent addition to the genus *Emaravirus* of plant viruses that also includes *European mountain ash ringspot-associated virus* (*EMARaV*), *Fig mosaic virus* (*FMV*) and *Rose rosette virus* (*RRV*) (McGavin et al., 2012; Mielke-Ehret & Mühlbach, 2012). Other, tentative, members of the genus *Emaravirus* are *Pigeonpea sterility mosaic virus* (*PPSMV*), *Pigeonpea sterility mosaic virus 2* (*PPSMV 2*), *Redbud yellow ringspot-associated virus* (*RyRSaV*), *High Plains wheat mosaic virus* (*HPWMoV*), *Wooly burdock yellow vein virus* (*WBYVV*), *Viburnum line pattern-associated virus* (*VibLPaV*) and *Blackberry leaf mottle-associated virus* (*BLaV*). Although not all of these viruses have been studied in the same detail, they all have multi-segmented genomes of (probably) negative-strand RNA, some are known to have roughly spherical particles enclosed in a double-membrane envelope and all have similarity in the amino acid sequences of some of their encoded proteins (Ebrahim-Nesbat & Izadpanah, 1992; Elbeaino et al., 2009, 2014, 2015; Laney et al., 2011; Sabanadzovic & Abou Ghanem-Sabanadzovic, 2011; Bi et al., 2012; Ishikawa et al., 2012; Tatineni et al., 2014). Other shared attributes are their transmission between plants by eriophyid mites (Mielke-Ehret & Mühlbach, 2012), and the presence of short conserved, imperfectly repeated nucleotide sequences at the 5’ and 3’ termini of the viral RNAs.

The virion complementary (VC) strand of each *RLBV* RNA encodes a single protein, i.e. RNA1 encodes a putative RNA-dependent RNA polymerase (RdRP), RNA2 encodes a polyprotein that is processed into two glycoproteins (GPs), RNA3 encodes a nucleocapsid (NC) protein, RNA4 encodes a movement protein (MP) (Ishikawa et al., 2013; Yu et al., 2013) and RNA5 encodes a P5 protein of unknown function. The RdRP, GP and NC proteins of emaraviruses have sequence and/or structural similarities with the equivalent proteins of bunyaviruses (Mielke-Ehret & Mühlbach, 2012), a large family of arthropod-transmitted, negative-strand RNA viruses that cause significant disease in humans, animals and plants. It is not yet clear what the complete genome complement for emaraviruses is or even whether the different emaraviruses have the same or different genome arrangements. So far no infectious clone systems have been produced for...
emaraviruses (or for any other negative-stand RNA plant viruses), and the low titre of these viruses in plants has made it difficult to reproducibly isolate and purify high-quality virus particles from plants for identification and characterization of viral RNAs.

Currently, HPWMoV has the largest known emaravirus genome, comprising eight RNAs (RNA1–8) together with an additional variant version of RNA3 (3B), encoding a NC protein with 11 % amino acid differences to the NC encoded by RNA3A (Tatineni et al., 2014). Six RNAs have been reported for FMV and PPSMV 2, five for PPSMV, and four for RRV, although it is likely that more RNAs remain to be discovered for these viruses.

Next-generation sequencing (NGS) of RNAs isolated from virus-infected plants is now the most usual method used for discovering emaravirus RNAs. As HPWMoV, FMV and PPSMV 2 have been shown to have more than five RNAs, we decided to re-examine the NGS data that was used to identify the five known RLBV RNAs (McGavin et al., 2012), and also do further virus sequencing, in an attempt to discover additional RNAs for RLBV.

RESULTS

The 454 sequencing data that was used previously to identify RLBV RNA1–5 was obtained from infected-plant dsRNA that provided an enrichment of virus sequences (McGavin et al., 2012). At that time, RNA1–4 could be identified by comparison of their encoded proteins with other viral proteins. RNA5, however, encoded a novel protein and was initially selected as a candidate RLBV RNA because of its high read count within the 454 sequence dataset and its extended assembled contig length. Thus far, all emaravirus VC RNAs have been characterized by the presence of a short (<100 nt) 5′ UTR, followed by an ORF >500 nt and an extensive 3′ UTR that is highly AT-rich. The assembled 454 sequence reads were scanned by eye to identify previously undetected contigs that did not align with any other known sequences, had a high read count, a size of >400 nt, encoded an ORF and included an AT-rich region. This analysis highlighted three additional contigs that might have been derived from previously undiscovered RLBV RNAs.

As confirmation, specific primers were designed to these contigs and shown to amplify sequences present in RLBV-infected plants, but absent in uninfected plants. The complete sequences of these new RLBV RNAs were determined by combining RNA-specific primers with the generic emaravirus 5′ and 3′ terminal primers T13(5) and T13(3). In addition, the precise 5′ and 3′ terminal sequences of these RNAs were determined by 5′ RACE PCR using dsRNA as the template as described previously (McGavin et al., 2012). Using these approaches it was possible to identify three previously unknown RLBV RNAs: RNA6–8.

Subsequently, attempts were made using the generic emaravirus 5′ and 3′ terminal primers to amplify complete RLBV RNAs for cloning and/or direct sequencing. In one experiment, three complete clones of RLBV RNA3, three of RNA7 and one of RNA6 were obtained and sequenced. In a second experiment, the amplified RNAs were directly sequenced without prior selection and cloning using Illumina NGS technology. Although RLBV RNA1 is 7062 nt and was not amplified as a discrete band by this technique, analysis of the assembled NGS reads showed that RNA1 sequences were present in both of two different datasets and that in one of the datasets (SMF3_S2) reads were obtained across the entire length of RNA1 (Table 1). In addition, in both datasets, the complete sequences of all the other seven RLBV RNAs (RNA2–8) were identified. However, the different RLBV RNAs were not equally represented either within each dataset or between the two datasets, e.g. in dataset SMF2_S1 RNA3, 6 and 7 had the highest number of sequence reads, whereas in dataset SMF3_S2 RNA2 and 5 had the highest read numbers. This most likely resulted from differences in the efficiencies of the PCR amplifications of the different cDNA samples which were done using slightly different primers; nevertheless, very high coverage of all eight RLBV RNAs was achieved in these experiments (Table 1).

Sequence diversity

Using reverse transcription (RT)-PCR, RNA6–8 were shown to be present in 11 different isolates of RLBV collected from commercially grown raspberry in both England and Scotland, wild raspberry from Scotland, and commercially grown raspberry from Poland and Finland. In addition, all eight RNAs were shown by RT-PCR to be present in individual raspberry plant samples. Examination of cloned RT-PCR products revealed sequence diversity between different isolates, particularly in the extended non-coding regions of RNA6–8. Analysis of the NGS data also revealed minor sequence differences located across RNA1–7, although no hotspots for sequence variation in these RNAs were apparent.

The analysis of RNA8 gave a more complicated result where, in addition to low-level variation in the 5′ part of the RNA, cloning of RT-PCR-derived RNA8 fragments identified a region of a much greater sequence diversity located downstream of nt 800 (Fig. 1).

This sequence variation makes it very difficult to derive a consensus sequence for this part of RNA8; however, two complete RNA8s (RNA8a and RNA8b; see below), which differ predominantly in their 3′ sequences, were assembled from different cloned RT-PCR products. The prevalence in field-grown plants of the different RNA8a and RNA8b 3′ sequences was examined further. Nine different raspberry samples from different locations in the UK (and one in Finland) were examined by RT-PCR using primers 2380 (5′-ATATGTTAGTTAGCTCAGTCAGT-3′) and 2376 (5′-ATATTTTTATTTATATTTATTTATTTAC-3′), which amplify
a 186 bp fragment from the 3’ UTR of RNA8a, and primers 2397 (5’-GATTATGCAGGGAATGTTATAGAAG-3’) and 2377 (5’-ACTTAATATATCATAACTTTAATTTATTCTTG-3’), which amplify a 244 bp band from the 3’ UTR of RNA8b. Five plants were found to contain both variants of RNA8 and three were found to have only RNA8a. One (UK) plant did not contain either sequence, but probably contained other RLBV RNA8 3’ UTR variant sequences that would require other primer sets to be amplified successfully.

NGS analysis revealed that although the majority of RNA8-specific sequence reads could be mapped to RNA8a there were multiple sequence variations located in the 3’ part of the molecule. In contrast, there were very few reads that mapped only to RNA8b in this region. This was evidence that RNA8a is much closer to a consensus sequence for RNA8 than is the second variant RNA8b.

**Sequence analysis**

The GenBank accession numbers for the RLBV sequences reported here are KP970121 (RNA6), P970122 (RNA7), KP970123 (RNA8a) and KP970124 (RNA8b).

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**Table 1. Coverage of RLBV RNA sequences in NGS datasets derived from raspberry cDNAs amplified using emaravirus-specific 5’ and 3’ terminal primers**

Minimum/maximum coverage numbers from mean coverage using a sliding window of 5 bp.

<table>
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<th>Reads map to</th>
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<th>No. mapped reads</th>
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**Fig. 1.** NGS reads mapped onto RNA8 highlighting the region of extreme heterogeneity after nt 800. Image derived from Tablet (Milne *et al.*, 2013); the RNA8a reference sequence runs horizontally with a scale bar (nt) added at the top and reads are shown as horizontal lines (additional read coverage cropped from bottom of figure for simplification). The read colour scheme shows mapped pairs in blue and green, with orphan reads mapped in red. Single base variations are in white, deletions are in dark blue and positions of insertions are in dark red. Most of the deletions and insertions cluster between nt 800 and 900.
RNA6 is 1095 nt in length, containing a 564 nt ORF, located in the complementary strand at nt 95–658, that encodes a 188 aa P6 protein of 22.3 kDa. A second, small ORF (nt 798–977, virus complementary strand) encodes a potential 59 aa P6b protein of 7.2 kDa.

RNA7 is 1089 nt, containing a 558 nt ORF, located in the complementary strand at nt 96–656, that encodes a 186 aa P7 protein of 21.95 kDa. A second, small, overlapping ORF (nt 634–792, virus complementary strand) encodes a potential 52 aa P7b protein of 6.2 kDa.

From the sequencing of several RLBV isolates, two variants of RNA8 were assembled. RNA8a, which likely represents the major variant of RNA8, is 1273 nt, containing a 699 nt ORF (nt 87–785, virus complementary strand), that encodes a 233 aa P8a protein of 21.16 kDa. The minor variant RNA8b is 1235 nt, containing a 699 nt ORF (nt 87–785, virus complementary strand), that encodes a 233 aa P8b protein of 21.4 kDa. RNA8 sequence variability extends into the 3’ part of the RNA8-encoded gene, so that different RNA8 variants encode P8 proteins with different C-terminal regions. Thus, the coding sequences of the P8a and P8b genes are 88.7 % identical at the nucleotide level. The amino acid sequences of the P8a and P8b proteins are 90 % identical (94 % similar), differing from one another in 22/233 residues, with 10 of these differences occurring at the C terminus of the P8 protein.

There is notable amino acid sequence similarity between the P6, P7 and P8 proteins (Fig. S1, available in the online Supplementary Material), with extended regions of identity occurring in the N-terminal regions (residues 1–129) of the three proteins. In this region, P6 and P7 are 43 % identical (68 % similar), P6 and P8 are 36 % identical (56 % similar), and P7 and P8 are 30 % identical (60 % similar). The coding sequences for these three proteins are not very highly conserved, with the nucleotide sequence identity between the two most highly related RNAs (RNA6 and 7) being only 57 %. Nevertheless, we assume that RNA6 and 7 have arisen perhaps by a duplication of an RNA8 progenitor, followed by deletion of some 3’ coding sequences and further mutational changes to produce RNA6 and 7.

Searches of sequence databases did not identify any similar proteins, even amongst other emaraviruses, and also did not identify any conserved amino acid domains that could suggest possible functions for the P6, P7 or P8 proteins.

**P6 and P7 enhance virus pathogenicity**

To examine whether the RLBV P6, P7 and P8 (P8a) proteins might have some detectable activity in plants, the RLBV genes were cloned individually into a potato virus X (PVX) infectious clone and inoculated by agro-infiltration to *Nicotiana benthamiana* plants. PVX expressing P7 caused strong symptoms of leaf mottling and malformation in systemic infected leaves (Fig. 2). PVX expressing P6 caused similar but less severe symptoms, whereas PVX expressing P8 caused no observable change in symptoms compared with a control construct of PVX expressing the GFP gene. Maintenance of the RLBV genes in PVX in the systemic leaves was confirmed by RT-PCR (data not shown). A Western blot of extracts of systemically infected leaves collected at 20 days post-infiltration revealed that levels of PVX coat protein were highest in plants infected with PVX P7 (1.45-fold increase) and also slightly higher (1.27-fold increase) in PVX-P6-infected plants compared with PVX-GFP-infected plants (Fig. 2). These results suggest that RLBV P7, and to a lesser extent RLBV P6, are pathogenicity factors that can enhance virus infection of *N. benthamiana*.

Some plant virus pathogenicity proteins are able to suppress RNA silencing (either local, systemic or both). Moreover, for some plant viruses that are actively transmitted by arthropod vectors and also replicate in their arthropod vector, such as tospoviruses (plant-infecting bunyaviruses), their pathogenicity proteins have been shown to interfere with the antiviral RNA silencing response, both in the plant host and arthropod vector (Takeda et al., 2002; Garcia et al., 2006; Schnettler et al., 2010). However, we were unable to detect any suppressor activity in arthropod cells or plants for any of these RLBV proteins (Figs. 3 and S2). In additional experiments, when these three RLBV proteins were transiently expressed in plants as GFP fusions, they all accumulated in the cytoplasm with no obvious subcellular/organellar localization (data not shown).

**Interactions between RLBV proteins**

To test for possible interactions between the RLBV proteins, yeast two-hybrid assays were performed for all the
cloned RLBV genes in all possible combinations, including self-interaction tests. The results of these experiments suggested the RLBV P3, P6 and P7 proteins were all capable of strong self-interaction. The P8 protein autoactivated the His reporter gene in yeast and so could not be assessed for interactions with itself or the other RLBV proteins using the yeast two-hybrid system.

As an alternative approach, a bimolecular fluorescence complementation (BiFC) analysis of the RLBV proteins, each fused at the C terminus with either the N-terminal YFP or C-terminal YFP, was performed. The fusion proteins were expressed in planta in N. benthamiana leaves. Interaction of the RLBV proteins was demonstrated by immunofluorescence microscopy to be present in the mite body at locations distant from the mouthparts and gut (Mielke-Ehret et al., 2010). This suggests EMARaV undergoes circulative transmission, with the possibility that the virus could replicate in the mite. It is likely that RLBV, including tomato spotted wilt virus, are able to replicate in their insect host (Wijkamp et al., 1993). At present there is no direct evidence that emaraviruses also can replicate in the mite vector; however, EMARaV was demonstrated by immunofluorescence microscopy to be present in the mite body at locations distant from the mouthparts and gut (Mielke-Ehret et al., 2010). This suggests EMARaV undergoes circulative transmission, with the possibility that the virus could replicate in the mite. It is likely that RLBV has a similar transmission mechanism to EMARaV and it is possible that one or more of these newly identified proteins is involved in this part of the virus life cycle. Although we were unable to show RNA silencing suppressor activity for proteins is surprising as it would suggest that they all have some shared activity or function. In our experiments P7, and to a lesser extent P6, had some effect on the pathogenicity of the heterologous virus PVX, suggesting that they could function similarly for RLBV. The bunyaviruses, including tomato spotted wilt virus, are able to replicate in their insect host (Wijkamp et al., 1993). At present there is no direct evidence that emaraviruses also can replicate in the mite vector; however, EMARaV was demonstrated by immunofluorescence microscopy to be present in the mite body at locations distant from the mouthparts and gut (Mielke-Ehret et al., 2010). This suggests EMARaV undergoes circulative transmission, with the possibility that the virus could replicate in the mite. It is likely that RLBV has a similar transmission mechanism to EMARaV and it is possible that one or more of these newly identified proteins is involved in this part of the virus life cycle. Although we were unable to show RNA silencing suppressor activity for...
any of the individual RLBV proteins here, further studies to investigate potential dsRNA or small RNA binding by these proteins, perhaps in combination, need to be performed.

METHODS

Plant infection and RNA extraction. RLBV was analysed from symptomatic leaves of wild and commercially grown raspberry plants. Total RNA was isolated using a RNeasy Plant Mini kit (Qiagen), according to the manufacturer’s instruction.

RT-PCR amplification and sequencing of RLBV RNAs. RT-PCR detection of the individual RLBV RNAs in infected plant was performed as described previously (McGavin et al., 2012) using RNA-specific primer sets (Table S1). For amplification of complete RLBV RNAs, the degenerate 5’ and 3’ terminal emaravirus primers T13(5) and T13(3) (Mielke & Muehlbach, 2007) were used in combination to prime RLBV cDNA synthesis, using total RNA isolated from infected plants as the template and SuperScript III reverse transcriptase (Invitrogen). The cDNA was amplified using the same primer pair and LA Taq (TaKaRa), with a programme of 94 °C 3 min for 1 cycle; 94 °C 15 s, 45 °C 30 s, 68 °C 2 min 30 s for 40 cycles; 68 °C 5 min for 1 cycle, producing clearly defined DNA fragments in a size range of ~1–2.5 kb. These were either excised from an agarose gel and cloned into pGemTeasy for sequencing or, in another experiment, the unprocessed amplification products were collected in bulk and submitted for NGS analysis using the Illumina MiSeq platform.

For the NGS experiment, total RNA was isolated from leaves of four separate raspberry plants showing symptoms of RLBV infection. cDNA was synthesized using either the combined T13(5)/T13(3) primers or primer PDAP213, which has the same emaravirus 3’ terminal sequence as T13(5) linked to a non-viral adaptor sequence (5’-GGGCAGCCGCTCGGTACCCCTAGTAGTGAACCTCC-3’; I. Tzanetakis, personal communication). The cDNA samples were amplified using the same primers in separate LA Taq reactions. Sample SMF2_S1 was derived from the RNAs of two different plants prepared using T13(5) and T13(3) primers for cDNA synthesis and PCR amplification. Sample SMF3_S2 was derived from the RNAs of two different plants prepared using the PDAP213 primer for cDNA synthesis and PCR amplification. The amplified cDNA was sheared using a Covaris M220 Ultrasonicator using recommended settings to a mean size of ~400 bp and converted to an Illumina sequencing-compatible DNA library using a TruSeq ChIP Sample Preparation kit (Illumina), before being sequenced with a 2 × 250 bp version 2 kit on an Illumina MiSeq machine.

Bioinformatic analysis of NGS data. Samples SMF2_S1 and SMF3_S2 were run on an Illumina MiSeq yielding 2 052 199 and 3 750 865 read pairs, respectively, of up to 251 bp each (European Nucleotide Archive accession numbers ERR964958 and ERR964959). Exploratory de novo assembly and mapping confirmed the presence of both known RLBV sequences, and host plant sequence including chloroplast, mitochondria and rRNA genes. The paired reads were mapped to RLBV RNA1–8a (excluding RNA8b) using the default settings of clc_mapper version 4.10.86742 from the CLC Assembly Cell command line tools version 4.1 (CLC bio). Read counts and coverage were calculated from the output BAM files using SAMtools (Li et al., 2009) (Table 1) and visually assessed in Tablet (Milne et al., 2013) (Fig. 1).

To assess the relative levels of RNA8a versus 8b, a separate mapping was performed against each RLBV RNA individually and a custom Python script used to identify properly mapped read pairs which mapped uniquely to each given RNA (https://github.com/peterjc/pico_galaxy/tree/master/tools/coverage_stats).

Expression of RLBV genes from PVX. The RLBV genes were PCR-amplified to add Gateway recombination sequences at their 5’ and 3’ termini, as recommended in the Invitrogen Gateway Technology

![Fig. 4. BiFC analysis of interactions between RLBV proteins: (a) P3/P3, (b) P6/P6, (c) P7/P7, (d) P8/P8, (e) P6/P3, (f) P8/P3, (g) P3/P7 (confocal image) and (h) P3/P7 (bright-field image). Bar, 50 μm.](http://jgv.microbiologyresearch.org)
Silencing suppression assay in Drosophila S2 cells. Drosophila melanogaster S2 cells were grown in Schneider’s media supplemented with 10% FCS, streptomycin (100 μg ml⁻¹) and penicillin (100 U ml⁻¹) at 28 °C. At 24 h pre-transfection, 5 x 10⁷ cells per well were seeded in a 96-well plate. Cells were co-transfected with copper sulphate-inducible constructs expressing firefly luciferase as a reporter (25 ng pMT-Fluc), Renilla luciferase as an internal control (6 ng pMT-Rluc) (van Rij et al., 2006) and constitutive expression constructs (150 ng p1B; Invitrogen) for maltose-binding protein (MBP) (negative control) (Schnettler et al., 2010). Drosophila C virus 1 A (DCViA; positive control) (Schnettler et al., 2010) or the RLBV proteins (GP/P2, NC/P3, P4, P5, P6, P7 and P8) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Silencing was induced at 48 h post-transfection by the addition of in vitro transcribed dsRNA (15 ng), either firefly luciferase-specific (dsFF) or EGFP-specific dsRNA (negative control), to the culture media. At 7 h post-dsRNA addition, luciferase expression was induced by addition of copper sulphate (final concentration 500 μM) and luciferase activity was measured 24 h later with the dual-luciferase assay (Promega) according to the manufacturer’s instructions. Silencing was measured by confocal microscopy.

BIFC analysis. The RLBV genes were cloned by Gateway recombination into each of two split-YFP binary vectors (pBAT-TL-B-sYFP-N and pBAT-TL-sYFP-C) (Uhrig et al., 2007) and transformed individually into A. tumefaciens GV3101. To examine potential interacting pairs, leaves of N. benthamiana were infiltrated with a 1 : 1 mixture of A. tumefaciens cultures (each at OD₅₉₀ 0.1) containing one N-YFP fusion construct and one C-YFP fusion construct. Infiltrated leaves were examined by confocal microscopy 2 days after infiltration.

Yeast two-hybrid interaction analysis. The RLBV genes were cloned by Gateway recombination into both a Gal4 DNA-binding domain (BD) bait vector (pAS-attR) and a Gal4 activation domain (AD) prey plasmid (pACT2-attR) (Uhrig et al., 2007). The clones were transformed separately into yeast strains AH109 (mating type a) and Y187 (mating type α), and combined in pairs by yeast mating. Positive interactions were identified by the growth of yeast colonies on selective media (–His–Leu–Trp). Stronger interactions were identified by the addition of 3 mM 3-amino-1,2,4-triazole to the selective plates. To test for possible autoactivation, the individual RLBV constructs, fused to the Gal4 BD or AD, were co-transformed into yeast with either an empty AD or BD vector plasmid, respectively, and the cells were plated onto –His selection medium.

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