Raspberry leaf blotch virus, a putative new member of the genus *Emaravirus*, encodes a novel genomic RNA

Wendy J. McGavin,1 Carolyn Mitchell,1 Peter J. A. Cock,2 Kathryn M. Wright1 and Stuart A. MacFarlane1

1Cell and Molecular Sciences Group, The James Hutton Institute (JHI), Invergowrie, Dundee DD2 5DA, UK
2Information and Computational Sciences Group, JHI, Invergowrie, Dundee DD2 5DA, UK

A new, segmented, negative-strand RNA virus with morphological and sequence similarities to other viruses in the genus *Emaravirus* was discovered in raspberry plants exhibiting symptoms of leaf blotch disorder, a disease previously attributed to the eriophyid raspberry leaf and bud mite (*Phyllocoptes gracilis*). The virus, tentatively named raspberry leaf blotch virus (RLBV), has five RNAs that each potentially encode a single protein on the complementary strand. RNAs 1, 2 and 3 encode, respectively, a putative RNA-dependent RNA polymerase, a glycoprotein precursor and the nucleocapsid. RNA4 encodes a protein with sequence similarity to proteins of unknown function that are encoded by the genomes of other emaraviruses. When expressed transiently in plants fused to green or red fluorescent protein, the RLBV P4 protein localized to the peripheral cell membrane and to punctate spots in the cell wall. These spots co-localized with GFP-tagged tobacco mosaic virus 30K cell-to-cell movement protein, which is itself known to associate with plasmodesmata. These results suggest that the P4 protein may be a movement protein for RLBV. The fifth RLBV RNA, encoding the P5 protein, is unique among the sequenced emaraviruses. The amino acid sequence of the P5 protein does not suggest any potential function; however, when expressed as a GFP fusion, it localized as small aggregates in the cytoplasm near to the periphery of the cell.

INTRODUCTION

Although *Rubus* plants are known to be susceptible to infection by more than 40 viruses and virus-like agents (Tzanetakis *et al.*, 2007), the symptoms caused by other pathogens and insect pests can be confused with those caused by viruses. This situation has been described for the infestation of raspberry and other *Rubus* species by the eriophyid mite *Phyllocoptes gracilis* (raspberry leaf and bud mite; RLBM). The feeding of this mite can cause yellow blotching as well as twisting of the leaves and distortion of the leaf margins (Gordon & Taylor, 1976), all of which are symptoms commonly associated with plant virus infection. The underside of the leaf with the blotch symptom has abnormal leaf-hair development, so that these areas appear pale green in colour compared with the greyish bloom of the remaining leaf parts. In addition, the terminal growing tip of the raspberry canes can be killed, affecting overall plant growth and development, and fruits may ripen irregularly and with reduced quality. Previously, a study was made of tayberry (a blackberry × raspberry hybrid) plants that were infested with RLBM and showed these symptoms (Jones *et al.*, 1984). Mechanical inoculation of extracts of the affected tayberry plants onto a range of herbaceous indicator plants did not reveal any underlying virus infection. Grafting from the tayberry plants to black raspberry indicator plants did result in apical tip necrosis, which can be a sign of virus infection, but further grafts to tayberry and other raspberry cultivars did not induce the RLBM-associated symptoms. Furthermore, the treatment of symptomatic tayberry plants with a systemic insecticide significantly reduced the symptoms in the first year and prevented their reappearance in the second year. Taken together, these results suggested that the severe symptoms that were seen in these affected plants, which were collectively referred to as raspberry leaf blotch disorder (RLBD), were caused by RLBM infestation and were not associated with a virus infection.

In recent years, RLBD has become an increasingly serious problem to raspberry growers, where it has particularly
affected the very popular cultivar Glen Ample, which is grown extensively under protective tunnels (HDC, 2009). We decided to revisit the issue of whether a virus is associated with RLBD, using molecular techniques that were not available to earlier researchers. From this work, we have discovered a new, multipartite, negative-strand RNA virus that we have tentatively named raspberry leaf blotch virus, and which we have detected in all raspberry plants showing RLBD symptoms, as well as in the raspberry leaf and bud mite itself.

RESULTS

Symptomatic RLBM-infested plants are infected with a new virus

The starting material for this work was obtained from a small farm in Fife, Scotland, where almost the entire population of raspberry plants (cv. Glen Ample) that were growing individually in pots inside a protective tunnel showed very severe symptoms of RLBD (Fig. 1). A buffer extract of highly symptomatic leaves was inoculated onto a range of herbaceous indicator plants, producing in Nicotiana benthamiana a systemically spreading infection with a clear yellowing of large sectors of the upper, infected leaves (Supplementary Fig. S1, available in JGV Online), but no symptoms on any of the other plant species.

Random amplification of cDNA derived from the raspberry dsRNA produced a 237 nt clone with sequence similarity (43 % identical after translation) to nucleocapsid (NC) protein sequences of a mite-transmitted virus, known variously as maize red stripe virus (MRSV), wheat mosaic virus or High Plains virus (Skare et al., 2006). This virus has sequence similarity to European mountain ash ringspot-associated virus (EMARAV), the type member of the genus Emaravirus, as well as to fig mosaic virus (FMV), rose rosette virus (RRV) and pigeonpea sterility mosaic virus (PPSMV), which are putative members of this genus (Kumar et al., 2003; Mielke & Muehlbach, 2007; Elbeaino et al., 2009a; Laney et al., 2011).

These results showed that the RLBD-affected plants carried a previously unidentified virus, which we have tentatively named raspberry leaf blotch virus (RLBV), that has similarities to EMARAV and the other, putative, emaraviruses.

Terminal sequences of the RLBV RNAs are related to those of other emaraviruses

The emaraviruses are segmented, negative-strand RNA viruses for which the 5’- and 3’-terminal sequences are nearly fully complementary for 13 nt (Mielke & Muehlbach, 2007; Elbeaino et al., 2009a; Laney et al., 2011). By combining primers derived from the RLBV NC gene cDNA clone and the conserved emaravirus terminal sequences (Supplementary Table S1, available in JGV Online), it was possible to amplify cDNA sequences totalling 1365 nt, encoding the complete RLBV NC gene and 5’ and 3’ non-coding regions.

The 5’- and 3’-terminal regions of the NC RNA, and subsequently all of the other RLBV RNAs, were amplified by RT-PCR of poly(A)-tailed or adaptor-ligated virus dsRNA using RNA-specific PCR primers (Supplementary Table S1) combined with primers recognizing the 3’-terminal RNA modifications (see Methods). This demonstrated that the terminal 13 nt of the authentic 5’ and 3’ ends of all of the RLBV RNAs were identical to those of the other sequenced emaraviruses.

Diagnostic primer design and transmission of RLBV

The bulk of the sequences of the RLBV RNAs were obtained by mass (Roche 454) sequencing of dsRNA-derived cDNAs. Gaps in the assembled sequence data were closed by RT-PCR amplification using sequence-specific primers. The completed sequences were used to design diagnostic PCR primers specific for each of the five virus RNAs, which were used in subsequent experiments (Supplementary Table S1).

Mechanical transmission of the virus from raspberry to N. benthamiana was achieved on many occasions, and on several occasions from N. benthamiana to N. benthamiana. Longer-term maintenance of the virus by sequential passaging was not successful, and storage of infected plant material at −20 or −80 °C prevented recovery of infectious virus.

Using RLBV-specific primer pairs, all five virus RNAs were detected by RT-PCR in symptomatic field-grown raspberry plants (Fig. 2), in N. benthamiana plants inoculated with extracts from infected raspberry, and in raspberry plants inoculated with mites in the glasshouse (data not shown). RLBV was also detected by RT-PCR amplification of RNA extracted from a bulked sample of mites collected from RLBD-affected plants (Fig. 2).

By laying pieces of mite-infested leaf onto leaves of healthy raspberry plants, we were able to transfer the mite onto the...
Partial purification of RLBV particles

A partial purification of RLBV particles from symptomatic raspberry and *N. benthamiana* leaves was achieved using the method of Kumar *et al.* (2003), which was used previously to purify PPMSV. Examination under the electron microscope of the material resulting from this purification procedure revealed indistinct, filamentous bodies similar to those reported for PPMSV (Kumar *et al.*, 2003) and MRSV (Skare *et al.*, 2006). When separated on a denaturing polyacrylamide gel, the virus preparation was found to contain two predominant proteins (Fig. 3a). An approximately 32 kDa protein was present in the virus preparation, but not in similarly processed fractions from healthy plants. Similar-sized proteins have been found in preparations of PPMSV, MRSV and EMARAV, and are suggested to be the virus NC protein that associates with the virus RNAs in a filamentous complex. The approximately 55 kDa protein is also present in fractions from healthy plants and is probably the RuBisCO large subunit. RT-PCR amplifications of RNA isolated from the purified particle preparation confirmed that all five virus (RLBV) RNAs were present in this material (data not shown).

Strand-specific RT-PCR

RNA was isolated from partially purified virus and amplified by RT-PCR targeting the virus RNA that encodes the RNA-dependent RNA polymerase (RDRP) protein. When the cDNA-synthesis reaction was primed using a primer complementary to the coding strand of the RDRP RNA, very little PCR amplification occurred (Fig. 3b). However, when the cDNA-synthesis reaction was primed using a primer complementary to the non-coding strand of the RNA, strong amplification occurred. When total RNA isolated from an infected *N. benthamiana* plant was used as a template, strong amplification of the RDRP gene fragment occurred regardless of which primer was used for cDNA synthesis, showing that both primers were competent to promote reverse transcription. It is known that primer-independent reverse transcription of virus RNAs, including those of bunyaviruses, which have some similarities to emaraviruses, can occur, making strand-specific RT-PCR free from non-specific contamination difficult to achieve (Tuiskunen *et al.*, 2010). However, our results indicate that RLBV particles contain minus-strand (non-coding) virus RNA, whereas both minus-strand and plus-strand virus RNAs are present in the total RNA fraction of infected leaves.
Sequence analysis of RLBV RNAs

We have identified five RLBV RNAs, four of which correspond to those found for the other emaraviruses and one of which is novel (Fig. 4). Each of the RNAs encodes a single large ORF located on the complementary strand of the genomic RNA. For EMARAV, RRV and FMV, the RNAs are numbered in order of decreasing size, with RNA3 encoding the NC and RNA4 encoding the P4 protein; however, for RLBV, both RNA4 and the novel RNA5 are larger than the NC RNA. The GenBank/EMBL accession numbers for the RLBV sequences are FR823299 (RNA1), FR823300 (RNA2), FR823301 (RNA3), FR823302 (RNA4) and FR823303 (RNA5).

RNA1 is 7062 nt in size and encodes a protein of 269 kDa that has sequence similarities (56–59 %) to the RDRPs of the emaraviruses RRV, EMARAV and FMV. It is clear from RDRP protein sequence analysis that RLBV sits in a well-supported clade with the other emaraviruses but, due to low sequence similarities between the different bunyavirus genera, other branches on the phylogenetic tree have only weak support (Fig. 5).

RNA2 is 2135 nt in size and encodes a 75 kDa protein that is related to the glycoprotein (GP) precursor proteins (46–49 % similarity) of EMARAV, RRV and FMV. The GP protein is predicted to have an N-terminal signal sequence and three transmembrane (TM) domains located between residues 129 and 142 (TM1), 185 and 205 (TM2), and 602 and 621 (TM3). The EMARAV, RRV and FMV GP proteins are predicted to be cleaved close to the C terminus of TM2 (at the sequence THADD for EMARAV, AKADD for RRV and ARADD for FMV) to produce a smaller N-terminal glycoprotein (Gn) and a larger C-terminal glycoprotein (Gc). However, the RLBV GP does not contain any similar sequence at or near to TM2 and its cleavage strategy remains to be identified.

RNA3 is 1365 nt in size and encodes the 32 kDa NC protein. The emaravirus NCs form a discrete group, with the RLBV NC being related most closely (56 % similar) to the MRSV NC, but only 44–47 % similar to NC proteins of EMARAV, RRV and FMV.

RNA4 is 1675 nt in size and encodes a 42 kDa protein that has very low (31 %) sequence similarity to the P4 protein of EMARAV and only 41 % similarity to the RRV and FMV P4 proteins. A potential signal sequence was identified at the N terminus of the P4 protein, suggesting that it may be a membrane-located protein.

RNA5 is 1718 nt in size and encodes a 56 kDa protein. The P5 protein has not been described for EMARAV, FMV or RRV, has no similarities to other proteins in the GenBank/EMBL sequence databases, and also has no identifiable amino acid sequence motifs that might suggest its function.

Localization of P4 and P5 fluorescent protein fusions

Initial observations of P4–GFP expression in leaves of N. benthamiana indicated that the protein was localized to the plasma membrane and to plasmodesmata (PD) (data not shown). The presence of P4 within PD was confirmed by co-localization of P4–mRFP and tobacco mosaic virus (TMV) 30K–GFP in PD of transgenic Nicotiana tabacum plants (Fig. 6a, b) P4–GFP was also shown to co-localize with aniline blue-stained callose within PD (data not shown). Co-expression of P4–GFP with a plasma-membrane marker, mOrange-LTI6b, demonstrated the localization of P4 in the plasma membrane (Fig. 6c). In contrast, P5–GFP was not membrane-associated, but formed small aggregated structures within the cytoplasm (Fig. 6d).

DISCUSSION

In this work, we have identified and characterized a new virus that is associated with a raspberry leaf blotch disorder previously attributed to the eriophyid mite P. gracilis. Currently, >40 samples of field-grown raspberry with RLBD symptoms, collected from various locations in Scotland, England and mainland Europe (Serbia), have been tested by RT-PCR and all have been found to contain RLBV, suggesting that the virus may be (part of) the cause of the symptoms associated with RLBD.

The virus was detected by RT-PCR in RNA extracts of RLBM, and transfer of mites from one plant to another resulted in co-transfer of the virus, suggesting that P. gracilis is the natural vector of RLBV. Similarly, the related viruses PPSMV, MRSV, EMARAV, RRV and FMV are all transmitted by eriophyid mites (Flock & Wallace, 1955; Seth, 1962; Amrine et al., 1988; Seifers et al., 1997; Mielke-Ehret et al., 2005; Seth, 1962; Amrine et al., 1988; Seifers et al., 1997; Mielke-Ehret et al., 2005).
The genome sequence of RLBV suggests that it should be considered as a new member of the genus *Emaravirus*. Notably, RLBV differs from the other sequenced *emaravirus*-in viruses in that it possesses five RNAs, whereas four RNAs have been reported for *EMARAV*, *RRV* and *FMV*, with no indication that other RNAs remain to be identified for these viruses. Although we have not examined all of our many RLBV-infected plant samples for the presence of RNA5, it has been found in every sample where we have done RT-PCR using RNA5-specific primers. These include multiple samples from different locations in Scotland, as well as samples from the south of England and from Serbia (south-central Europe). These results suggest that RNA5 is a ubiquitous component of the RLBV genome. Only a single sequence, representing part of the NC RNA, has been reported for both MRSV and PPSMV; however, between five and seven RNAs were apparent when different PPSMV preparations were analysed by agarose-gel electrophoresis (Kumar et al., 2003) and when cDNA clones of MRSV were compared (Skare et al., 2006). More work is required to definitively describe the genome structures of these viruses. Variation in genome-segment number is also found with negative-strand RNA viruses in the genus *Tenuivirus*, where, for example, rice grassy stunt virus has six RNAs, maize stripe virus has five RNAs and rice stripe virus has four RNAs. It is possible that the genome structure of *emaraviruses* is similarly variable.

The RLBV P4 protein contains a signal-peptide sequence at its N terminus and, when fused at the C terminus to GFP or mRFP, it localized to the plasma membrane. In addition, some of the pool of P4–GFP was localized to discrete spots in the cell wall and co-localized with the P3-targeting TMV 30K protein. This is circumstantial evidence that the P4 protein may be involved in cell-to-cell movement of RLBV. The FMV and RRV P4 proteins, which are of a similar size (40–41 kDa) and have approximately 41% sequence similarity to the RLBV P4, are not predicted to contain a signal peptide or other TM domain. Likewise, the EMARAV P4 protein, which is much smaller in size (27 kDa), also does not contain a signal-peptide domain. It is possible that the RLBV P4 protein that is located in the plasma membrane may have an additional function that is not required by EMARAV, RRV and FMV and is separate from virus movement.

The RNA5 and its encoded protein P5 are, at present, unique to RLBV. Sequence analysis of the P5 protein did not identify any functional motifs, including any potential TM domains. In transient expression experiments, the
Fig. 6. Localization of P4 and P5 proteins expressed in Nicotiana leaf epidermis. (a) P4–mRFP (magenta) in the plasma membrane and co-localized with TMV 30K–GFP (green) to plasmodesmata (PD) (arrows, overlay white), in epidermal cells of transgenic N. tabacum. (b) A single section showing the co-localization (left panel) of P4–mRFP (centre panel, magenta) and TMV 30K–GFP (right panel, green) to PD (arrows); P4 can also be seen in the plasma membrane. (c) Single-section image of N. benthamiana epidermis, showing P4–GFP (magenta, centre panel) in PD (arrows), and co-localized (left panel) with mOrange–LTi6b (green, right panel) to the plasma membrane (arrowheads). (d) P5–GFP localized to small aggregate structures within the cytoplasm of N. benthamiana epidermal cells. The plasma membrane can be seen labelled with mOrange–LTi6b (green). Bars, 50 μm (a, d); 5 μm (b, c).

P5–GFP fusion protein was localized in aggregated structures in the cytoplasm; however, further work is required for the identification of the role of P5 in the virus life cycle.

**METHODS**

**Plant material.** Symptomatic leaves of RLBV-infested raspberry (cv. Glen Ample) were collected from plants grown under protection (in plastic tunnels) at JHI, ScotHall Farm, Fife, Scotland, and in the field at Blairgowrie, Scotland. The leaves were homogenized in 2% nicotine, and the extract was inoculated manually onto leaves of N. benthamiana, Chenopodium quinoa, Chenopodium amaranthicolor and Nicotiana clevelandii plants that had been dusted with abrasive corundum powder.

**RNA extraction from plants.** Total RNA was extracted from symptomatic raspberry leaves using the method of Thompson et al. (2003), and from N. benthamiana leaves using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s instructions. dsRNA was extracted from symptomatic raspberry and N. benthamiana leaves using a method described previously (McGavin et al., 2011).

**Initial cloning and sequencing.** cDNA was synthesized from dsRNA extracted from symptomatic raspberry leaves using random hexamer primers and ThermoScript reverse transcriptase (Invitrogen), followed by a combined RNase H and Superscript II (Invitrogen) treatment based on the method of Tzanetakis et al. (2005). The double-stranded cDNA was A-tailed by treatment with Taq polymerase and cloned directly, without amplification, into the pCR-TOPO-TA vector (Invitrogen), following the manufacturer’s instructions. Plasmid DNA was isolated from individual clones and sequenced at the JHI DNA sequencing facility.

**Virus-particle purification.** RLBV-infected N. benthamiana or raspberry leaves (200 g) were extracted in 400 ml HB buffer [0.05 M Tris/HCl (pH 8.0), 1% thioglycerol, 0.02 M EDTA, 0.25 M sodium sulphite, 1% polyvinylpyrrolidone, 0.02 M sodium diethyldithiocarbamate]. The homogenate was squeezed through muslin, filtered by centrifugation at 5000 r.p.m. for 4 min in a Sorvall rotor F14S. The resultant pellet was resuspended in 100 ml 1/10-diluted HB buffer and stored overnight at 4°C. The supernatant was clarified by an equal volume of chloroform. The aqueous phase was collected and centrifuged at 48 000 r.p.m. for 1 h in a Beckman rotor 50.2Ti. The pellet was resuspended in 1 ml 1/10-diluted HB buffer. After a short, low-speed centrifugation (5000 r.p.m., 10 min, Eppendorf microcentrifuge 5415R), the supernatant was loaded onto a 20% sucrose cushion and centrifuged at 40 000 r.p.m. for 2 h in a Beckman rotor SW41. As a final step, the collected pellet was resuspended in 1 ml sterile water.

One-third of the virus preparation was analysed by SDS-PAGE and Coomassie blue staining. RNA was extracted from the remaining two-thirds of the preparation, by adding an equal volume of RNA-extraction buffer [0.01 M Tris/HCl (pH 7.6), 0.05 M NaCl], and 0.2 vol. 10% SDS. The mixture was mixed thoroughly then extracted twice with phenol. The RNA was precipitated with 2.5 vols ethanol and 0.1 vol. 3 M sodium acetate.

To determine which strand of virus RNA was present in the partially purified virus preparation, cDNA was synthesized using a primer complementary to either minus-strand RNA (primer no. 1499) or plus-strand RNA (primer no. 1500) (Supplementary Table S1). Subsequent PCR amplification of part of the RDRP gene used the same two primers in combination.

**Identification of terminal sequences.** A poly(A) tail was added to the RLBV dsRNA isolated from N. benthamiana using poly(A) polynucleotides at 37°C for 10 min. After phenol extraction and ethanol precipitation, the A-tailed dsRNA was annealed to the de-adaptor primer (1096: GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT) by heating at 99°C for 5 min and snap-cooling in ice. cDNA was synthesized using a SuperScript II kit (Invitrogen) and incubation at 55°C for 1 h. PCR amplification of the terminal regions of the RLBV RNAs used primer 1097 (GACTCGAGTCGACATCG) in combination with RLBV RNA-specific primers (Supplementary Table S1).

A second approach used the method of Coutts & Livieratos (2003), in which a DNA oligonucleotide with its 3’ end blocked by the addition of deoxyoctosine (Oligo1rev; GATCCACTAGTCTAGAGCGGCGGdC) was ligated to the 5’ ends of dsRNAs extracted from RLBV-infected N. benthamiana. The dsRNA and primer were heated to 99°C and snap-cooled, then added to 70 μl ligase buffer, together with 10 μl DMSO, 1 μl RNase inhibitor and 2 μl T4 RNA ligase 2 (New England Biolabs), before incubation for 16 h at 17°C. Unligated oligonucleotide was removed using a spin column (Millipore), then the RNA was precipitated and washed with 70% ethanol. cDNA was synthesized using a SuperScript II kit and a primer (Oligo2for; GCCGCTCTAGAATCTAGTGATCG)

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complementary to Oligo1rev. Subsequently, the Oligo2for primer was used in combination with RLBV RNA-specific primers for PCR amplification of the terminal regions of the RLBV RNAs (Supplementary Table S1). The amplified products were cloned into pGEM-T Easy (Promega) and sequenced.

**RT-PCR detection of RLBV sequences in plants, purified virus preparations and mites.** All RT-PCRs were done using RT-PCR beads (Ready-To-Go; GE Healthcare), where the RNA samples were reverse-transcribed at 42 °C for 1 h prior to PCR amplification. RLBV sequences were amplified using both total RNA and dsRNA samples isolated from raspberry and *N. benthamiana*, and also RNA extracted from purified virus particles, using sequence-specific primers that were designed from cDNA clones and Roche 454 sequence data (Supplementary Table S1).

For detection of RLBV in *P. gracilis*, mites were viewed on the underside of infested raspberry leaves using a low-power stereo microscope and collected individually with a tool made from a single human eyelash as described by Skare et al. (2003). Twenty mites were collected together in a single microcentrifuge tube and RNA was isolated from them using an RNaseasy mini kit (Qiagen) and eluted in 50 μl water. For detection of RLBV, 3 μl mite RNA was amplified using primers 1287 and 1095 that were derived from the RLBV RNA NC sequence. As we do not have a non-viruliferous population of mites, RNA for use as an RT-PCR negative control was extracted, by the same method, from approximately 50 healthy aphids (small raspberry aphid, *Aphis idaei*).

**Roche 454 sequencing.** Nucleic acid recovered from plants using the dsRNA-isolation procedure was quantified using the NanoDrop system (Thermo Scientific) to be between 4.5 and 0.6 ng μl⁻¹. For reverse transcription, 9.5 μl dsRNA (42–57 ng) was mixed with 50 pmol primer 1088 (5′-GACGTCCAGATCGCGATTTCNNNNNN-3′) in a 12 μl volume, and cDNA synthesis was done as described previously (McGavin et al., 2011). PCR amplification used the adaptor primer 1089 (5′-GACGTCCAGATCGCGATTTC-3′), also as described previously (McGavin et al., 2011). At the University of Liverpool Centre for Genomic Research (Liverpool, UK), 5 μg amplified DNA was barcoded (Roche MID4, AGCACTGTAG) and then sequenced on a GS FLX Titanium Series machine (Roche). The reads were then assembled de novo using Roche ‘Newbler’ assemblies v. 2.0.01.14, with the primer 1089 sequence and its reverse complement specified for vector screening. All of the derived contigs were then searched against a local copy of the NCBI non-redundant protein database using BLASTX to identify any potential matches with other viral proteins for further examination. The highlighted contigs and further capillary sequence data were assembled manually to give the five RLBV RNA sequences.

**Bioinformatic analyses.** Software for protein-structure prediction (InterProScan, TMPred, SignalP) and similarity searches (BLAST) were accessed at the European Bioinformatics Institute (http://www.ebi.ac.uk/). Protein sequence-similarity determinations were made using the programme MatGAT, with the BLOSUM62 alignment matrix and default settings for gap insertion (Campanella et al., 2003).

Amino acid sequence alignments were generated using MUSCLE v. 3.8.31 (Edgar, 2004) with sequences from RLBV, other emaraviruses and representative members of other genera of negative-strand RNA viruses from plants and animals, using *Sonchus* yellow net virus (SYNV) as an outgroup. TOPALi v. 2.5 and RAXML v. 7.0.4 were used for phylogenetic model selection and tree generation, using both Bayesian and 1000 replicate-bootstrapped maximum-likelihood methods (Stamatakis et al., 2005; Milne et al., 2009).

**GFP and mRFP fusions and confocal microscopy.** The predicted ORFs for the RLBV P4 and P5 proteins were PCR-amplified to add Gateway-compatible (Invitrogen) flanking sequences and recombined into the binary vectors pBAT-KL-GFP (Canto et al., 2006) and pMDC83 (Curtis & Grossniklaus, 2003) to create C-terminus fusions of GFP or mRFP, respectively, to the virus proteins.

The GFP fusions were expressed in leaves of *N. benthamiana* using *Agrobacterium tumefaciens* strain AGL1 either singly or co-expressed with *A. tumefaciens* strain LBA4404 expressing a plasma-membrane marker, mOrange-LTI6b (Vidaurre et al., 2007). mRFP fusions were expressed in leaves of *N. tabacum* expressing a PD marker, TMV 30K movement protein fused to GFP (35 S::TMV 30K–GFP) (Reichel & Beachy, 2000). The subcellular location of each fusion, expressed in the abaxial epidermis, was examined using a Leica TCS-SP2 AOBS confocal laser-scanning microscope (Leica Microsystems) fitted with a Leica HCX APO ×63/0.9 W water-dipping lens. GFP was imaged singly or sequentially in combination with mRFP or mOrange: GFP excitation 488 nm, emission 500–530 nm; mRFP or mOrange excitation 561 nm, emission 590–630 nm. Images are presented as either single sections or as maximum-intensity projections of multiple-layered stacks. Images were assembled and edited using Adobe Photoshop v. 8.0.

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