A +1 ribosomal frameshifting motif prevalent among plant amalgaviruses

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(Article begins on next page)
A +1 ribosomal frameshifting motif prevalent among plant amalgaviruses

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1. Introduction

Family Amalgaviridae is a recently recognized taxon that currently comprises four species of plant viruses (Blueberry latent virus, Rhododendron virus A, Southern tomato virus, and Vicia cryptic virus M) in one genus (Amalgavirus) (Adams et al., 2014; Liu and Chen, 2009; Martin et al., 2011; Sabanadzovic et al., 2009, 2010). These plant amalgaviruses have small dsRNA genomes (3427–3437 bp) and have not yet been shown to form filamentous nucleocapsid (NC) protein (Krupovic et al., 2015), or replication factory matrix-like protein (Isogai et al., 2011)) and an ORF1 +2-encoded fusion protein sequence. Instead, they are transmitted vertically through seeds and are thought unlikely to be capable of efficient extracellular transmission, unless possibly by vector. The genomic plus strands of plant amalgaviruses encompass two partially overlapping long open reading frames (ORFs), with downstream ORF2 overlapping ORF1 in the +1 frame. They are thereby thought to encode only two proteins, an ORF1-encoded product of unknown specific function (though potential icosahedral capsid protein (CP)), filamentous nucleocapsid (NC) protein (Krupovic et al., 2015), or replication factory matrix-like protein (Isogai et al., 2011)) and an ORF1 +2-encoded fusion protein that is translated consequent to +1 programmed ribosomal frameshifting (PRF) (Depierreux et al., 2016; Firth et al., 2012; Liu and Chen, 2009; Martin et al., 2011; Sabanadzovic et al., 2009, 2010). The ORF2-encoded portion of this fusion protein is indicated by conserved sequence motifs to be the viral RNA-dependent RNA polymerase (RdRp).

For the current report, we undertook studies to identify novel plant amalgavirus sequences, with the goal of learning more about these viruses through sequence comparisons. Liu et al. (2012) searched the Expressed Sequence Tags (EST) database at GenBank/EMBL/DDJB for amalgavirus-like sequences and identified partial sequences (268–2127 nt in length) from 7 different plant species. We searched instead the Transcriptome Shotgun Assembly (TSA) database at GenBank/EMBL/DDJB in an effort to identify more complete sequences. Here we report the complete protein-coding sequences of 16 proposed new amalgaviruses, derived from 12 different plant species, plus the nearly complete protein-coding sequences of 3 others. Detailed examinations of these sequences provided several new insights as described below.

2. Results

Using the predicted ORF1 +2-encoded fusion protein sequence of blueberry latent virus (BLV) (GenBank YP_003934623) as query for a tblastn search of the TSA database for plants (NCBI taxonomic identifier 3193), we identified 37 TSA accessions with E-value scores of 0.0, indicating strong sequence similarities, and lengths

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E-mail addresses: mnibert@hms.harvard.edu (M.L. Nibert), jessepyle@g.harvard.edu (J.D. Pyle), aef24@cam.ac.uk (A.E. Firth).
Some of the E. coli-like sequences appear to be truncated at one or both ends. The lengths are calculated from the respective terminus (i.e., is not flanked by 1 PRF site) to the first in-frame stop codon. For ORF1 translation products that appear to be truncated at one or both ends, the lengths are calculated to the termini and are listed in parentheses.

\[ \text{orf1p(aa)} \times \text{orf2p(aa)} = \text{orf1+2p(aa)} \]

**Table 1**

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<th>Putative host species (cultivar)</th>
<th>GenBank accession no.</th>
<th>Algalamavirus (abbrev.)</th>
<th>Length (bp)</th>
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<th>ORF2p (aa)</th>
<th>ORF1+2p (aa)</th>
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<td>VCVA1</td>
<td>3434</td>
<td>394</td>
<td>771</td>
<td>1057</td>
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</table>

* Sequences that appear to be truncated at one or both ends have their lengths listed in parentheses.
* For apparently full-length ORF1 translation products, the lengths are calculated from the first residue following the proposed +1 PRF site to the first in-frame stop codon. For ORF2 translation products that appear to be truncated at the C-terminal end, the lengths are calculated from the first residue following the proposed +1 PRF site to the C-terminus and are listed in parentheses.
* For apparently full-length ORF1+2 translation products, the lengths are calculated from the first in-frame Met residue in ORF1p to the respective termini, taking into account the proposed +1 PRF site.
* Sequences for which peer-reviewed papers are also available, as indicated in the text.
* Sequences that were extended by reassembling contigs from SRA entries (see text and Table S1).

Between 2793 and 3478 nt, approximating the genome lengths of previously characterized plant algalamaviruses (Table 1, bottom). Some of the E=0.0 accessions derived from the same plant species (Allium cepa and Lolium perenne) and were nearly identical to one another (>99% identity), so that after the shorter among these replicates were also excluded, we were left with a set of 19 distinct TSA accessions for further study (Table 1, top). Using the predicted ORF1+2-encoded fusion protein sequences of the other previously characterized plant algalamaviruses as queries in tblastn searches of the TSA database for plants did not expand this list of E=0.0 accessions.

Do these 19 TSA accessions represent the nearly complete genome sequences of novel plant algalamaviruses? Strikingly, as in previously characterized plant algalamaviruses, the apparent plus-strand sequence of each of these accessions contains two partially overlapping long ORFs, with downstream ORF2 overlapping ORF1 in the +1 frame. The lengths of the ORF1–ORF2 overlap regions in the sequences range from 287 to 968 nt, compared with 293–611 nt in previously characterized plant algalamaviruses. Also strikingly, in the overlap regions of the sequences except the one from Capsicum annuum, and positioned in the proper reading frame in each sequence, is found the putative +1 PRF motif UUU_CGN (underline, codon boundary for ORF1; N, any nucleotide; CGN, a rare Arg codon) (Fig. 1A), which has been shown to promote translation of the influenza A virus PA-X protein (Firth et al., 2012; Jagger et al., 2012) and also recently proposed to allow ORF1+2-encoded fusion protein translation by plant algalamaviruses (Firth et al., 2012) and the algal-like mycoviruses Zygosaccharomycetes bailii virus Z (ZbV-Z) (Depierreux et al., 2016). This finding suggests to us the strong likelihood that the ORF2 product encoded by each of the 19 TSA accessions is translated as part of an ORF1+2-encoded fusion protein consequent to +1 PRF at the position of the proposed motif (Fig. 1A). The proposed motif for +1 PRF in the TSA accession from C. annuum is analyzed in Discussion.

As we were performing the preceding analysis, we noted that in 7 of the 19 TSA accessions, ORF1 and/or ORF2 remains open to the respective nucleotide sequence terminus (i.e., is not flanked by one or more stop codon) and encodes a smaller-than-expected protein product (Table 1, top). These 7 sequences hence appear to be partially truncated with respect to their protein-coding regions. In an effort to correct this situation, we turned to data sets in the Sequence Read Archive (SRA) database at NCBI, which were accessible for each of these TSA accessions. By examining the SRA data sets and incorporating additional reads into the transcript contigs, we were able to extend the lengths of 5 of the TSA accessions (GenBank GAYX01076418, GBXZ01009138, GCJW01039808, GEAC01063629, and GEEOC01025317), for 4 of them such that their protein-coding regions are no longer truncated (Table 1, top). As a result, the protein-coding regions of only 3 of the 19 TSA accessions appear to remain truncated at one or both termini (GenBank GAMH01005363, GBIE01028534, and GEEOC01025317). See Table S1 for reassembly information for the 5 extended sequences and Data S1 for the reassembled sequences themselves.
plant amalgaviruses are also shown, along with the consensus at bottom. Both UUU
A site for continued translation. (B) Previously proposed motif from plant amalgavirus STV (Depierreux et al.,
seen in PSI-BLAST searches of the Non-redundant Protein Sequences
database, each was found to be highly similar to the ORF2p (RdRp)
sequences that encompass complete protein-coding regions span
ORF1-, ORF2-, and ORF1
1077 aa), very similar to
1071 aa), very similar to
1058 aa), very similar to
1048 aa), very similar to
1044 aa), very similar to
1037 aa), very similar to
1034 aa), very similar to
1025 aa), very similar to
1008 aa), very similar to
990 aa), very similar to
974 aa), very similar to
956 aa), very similar to
937 aa), very similar to
925 aa), very similar to
911 aa), very similar to
897 aa), very similar to
881 aa), very similar to
867 aa), very similar to
853 aa), very similar to
839 aa), very similar to
825 aa), very similar to
811 aa), very similar to
797 aa), very similar to
789 aa), very similar to
775 aa), very similar to
769 aa), very similar to
763 aa), very similar to
753 aa), very similar to
739 aa), very similar to
725 aa), very similar to
711 aa), very similar to
697 aa), very similar to
681 aa), very similar to
667 aa), very similar to
653 aa), very similar to
639 aa), very similar to
625 aa), very similar to
611 aa), very similar to
597 aa), very similar to
583 aa), very similar to
569 aa), very similar to
555 aa), very similar to
541 aa), very similar to
525 aa), very similar to
511 aa), very similar to
497 aa), very similar to
483 aa), very similar to
469 aa), very similar to
455 aa), very similar to
441 aa), very similar to
425 aa), very similar to
411 aa), very similar to
397 aa), very similar to
383 aa), very similar to
369 aa), very similar to
355 aa), very similar to
341 aa), very similar to
325 aa), very similar to
311 aa), very similar to
297 aa), very similar to
283 aa), very similar to
269 aa), very similar to
255 aa), very similar to
241 aa), very similar to
225 aa), very similar to
211 aa), very similar to
197 aa), very similar to
183 aa), very similar to
169 aa), very similar to
155 aa), very similar to
141 aa), very similar to
127 aa), very similar to
113 aa), very similar to
99 aa), very similar to
85 aa), very similar to
71 aa), very similar to
57 aa), very similar to
43 aa), very similar to
29 aa), very similar to
15 aa), very similar to

Fig. 1. Motifs for +1 PRF. Anticodon:codon base pairs are indicated by filled circles. The positions of these +1 PRF motifs in a broader, aligned RNA sequence context are shown in Fig. S3. (A) Previously identified motif from influenza (Flu)A virus segment (S3) and previously proposed motifs from plant amalgaviruses BLV, RRV-
A, and VCV-M (Firth et al., 2012) are shown. Proposed motifs from newly proposed plant amalgaviruses are also shown, along with the consensus at bottom. Both UUU and UUC are decoded by a single tRNAiso-acceptor that has anticodon 3’AAG (Grosjean et al., 2010). First positioned on codon UUU in the +1 PRF motif, this tRNA is then thought to slip forward by one nucleotide (arrow) in the P site (onto codon UUC), positioning the next codon (CUU) in the A site for continued translation. (B) Previously proposed motif from plant amalgavirus STV (Depierreux et al., 2016) is shown. Anticodon 3’UCC (first positioned on codon AGG in the motif), was suggested to slip forward by one nucleotide in the P site (onto codon AGC), positioning the next codon (UGC) in the A site for continued translation. (C) Newly proposed motifs from plant amalgaviruses CaAV1 and STV are shown. Anticodon 3’AAG (first positioned on codon CUU in the motif) is thought to slip forward by one nucleotide in the P site (onto codon UUG), positioning the next codon (CUU) in the A site for continued translation.

Table 1 includes the protein lengths of the ORF1-, ORF2-, and ORF1+2-encoded translation products deduced from the 19 TSA-derived amalgavirus-like sequences as well as from the four originally characterized plant amalgaviruses. Notably, the ORF1-, ORF2-, and ORF1+2-encoded protein lengths deduced from the 16 sequences that encompass complete protein-coding regions span narrow ranges (ORF1p, 375–403 aa; ORF2p post-fameshifting sequences, 769–787 aa; ORF1+2p, 1048–1071 aa), very similar to those spanned in the original plant amalgaviruses (ORF1p, 375–404 aa; ORF2p post-fameshifting sequences, 771–789 aa; ORF1+2p, 1054–1077 aa) (Table 1). These protein lengths deduced from the other 3 TSA-derived amalgavirus-like sequences are generally smaller, consistent with their partial truncation at one or both ends, probably due to incomplete sequencing.

When the 19 deduced ORF2p sequences were used as queries in PSI-BLAST searches of the Non-redundant Protein Sequences (NR) database, each was found to be highly similar to the ORF2p (RdRp) sequences of originally characterized plant amalgaviruses (E-values, 0.0). As another way to address the degrees of similarity among these proposed and original plant amalgaviruses, we performed pairwise alignments. The pairwise identity scores for their separate ORF1 and ORF2 products are shown in Fig. 2 and provide further evidence that they are all closely related, especially as reflected by the scores for ORF2p (RdRp). Some pairs are especially closely related, namely, Capsicum annuum amalgavirus 1 (CaAV1) and STV, MsAV1 and VCV-M, AoAV1 and FpAV1, and FpAV3 and LpAV1 (See Table 1 for other abbreviations). Interestingly, in each of these four pairs, the sequences originated from plants of the same taxonomic family and subfamily: CaAV1 and STV, Solanaceae/Solanoidae; MsAV1 and VCV-M, Fabaceae/Fabioideae; AoAV1 and FpAV1, Poaceae/Pooidae; and FpAV3 and LpAV1, Poaceae/ Pooidae. These latter findings are consistent with coevolution of amalgaviruses with their respective plant hosts.

The 19 deduced ORF2p (RdRp) sequences were next compared by phylogenetic methods. The sequence set for these studies included not only the proposed and original plant amalgaviruses but also a number of viruses whose RdRp sequences have been previously noted to be related to them: ZbV-Z (Depierreux et al., 2016), monosegmented viruses from proposed genus Unirnavirus (Jiang et al., 2015; Koloniuk et al., 2015; Kotta-Loizou et al., 2015; Lin et al., 2015; Nerva et al., 2015; Zhu et al., 2015); viruses related to CTVT, which are presumably all bisegmented (Botella et al., 2015; Márquez et al., 2007; Vainio et al., 2012; Yu et al., 2009; Zheng et al., 2013); and representative bisegmented viruses from family Partitiviridae (Nibert et al., 2014) (see Table S2 for abbreviations and GenBank numbers for the additional viruses; RdRp is generally encoded on RNA1 of the bisegmented viruses). Sequences were aligned using MAFFT (Katoh et al., 2013) and then used for maximum-likelihood phylogenetic analyses using PhyML (Guindon et al., 2010) with the LG or rtREV substitution model for amino acids. The resulting RdRp-based trees provided consistent strong evidence that the proposed and original plant amalgaviruses all cluster together in the same taxon (Fig. 3), corresponding to approved genus Amalgavirus. Amalgala-like mycovirus ZbV-Z is next most closely related to this taxon (Fig. 3), consistent with previous findings (Depierreux et al., 2016; Koloniuk et al., 2015).

Multiple sequence alignments for ORF2p from proposed and original plant amalgaviruses were also examined in detail for conserved residues including known RdRp motifs (Poch et al., 1989; Koonin, 1991; Bruenn, 2003). The 795-position alignment generated using MAFFT appears notably robust in terms of including gaps at only 7 positions other than in the terminal regions, in having 136 positions (17%) that are wholly conserved among the 21 ORF2p sequences included in this comparison, and in having 451 positions in the consensus (57%) that are at least similar among all 21 of the sequences (Fig. S1). RdRp motifs A, B, and C (or IV, V, and VI) are especially easy to spot in the consensus and occur in the usual order: A, 341-sshELDwKFDfnRP-352; B, 406-hpGMvPscSltWTGhshTuhNhY-426; and C, 445-CAGDdNLT-454 (h, hydrophobic; n, negatively charged; p, polar; s, small; t, turn-like; u, tiny). There are also regions of strong sequence conservation near the C-terminus of ORF2p, seemingly beyond the central region of conserved RdRp motifs (Fig. S1, Fig. 4A), suggesting that another conserved function might be mediated by these C-terminal sequences. A large central portion of the MAFFT alignment is nearly identical with one generated using PROMALS3D, which additionally predicts a consensus secondary structure comprising a mixture of α-helices and β-strands (Fig. S1).

Multiple sequence alignments for ORF1p from proposed and original plant amalgaviruses were also examined in detail for conserved residues. As expected from the pairwise scores (Fig. 2), the 413-position alignment generated using MAFFT shows a much lower degree of conservation than the alignment for ORF2p,
Fig. 2. Pairwise sequence identity scores. Sequences of the ORF1 (lower left) and ORF2 (upper right) translation products of the indicated viruses (original and proposed) were compared in pairs using EMBOSS: needle or needleall. Sequence identity scores are shown in %. Shading off the diagonal highlights more closely related pairs for which the ORF1p score is \( > 40 \% \) and the ORF2p score is \( > 65 \% \). For these analyses, the ORF1p sequences of AoAV1 and PpAV1 began with the first residue instead of the first Met residue since their encoding sequences appear to be 5′-truncated, and the ORF2p sequences of AoAV1 and SeAV1 ended with the last residue instead of the last residue before the downstream stop codon since their encoding sequences appear to be 3′-truncated; as a result, their scores here may be artificially low in some instances.

Fig. 3. Phylogenetic tree, ORF2p (RdRp). Sequences of the ORF2 translation products were aligned using MAFFT and then subjected to phylogenetic analysis using PhyML as described in Materials and Methods. Values estimated from the data were Proportion of invariable sites, 0.010, and Gamma shape parameter, 1.473. Alternative use of the rtREV amino acid substitution model for PhyML (in place of LG) yielded results largely identical to those shown here. Proposed plant amalgaviruses new to this report are labeled in gray. The tree is displayed as a rectangular phylogram rooted on the branch to family Partitiviridae members. Branch support values are shown in %, and those with support values \( < 50 \% \) are collapsed to the preceding node. The few branches with support values between 50% and 80% are drawn with thinner lines. Scale bar, average number of substitutions per alignment position. See Table S2 for a summary of abbreviations and GenBank numbers. Vertical lines: approved or proposed spans of genera and families (family Amalgaviridae has been proposed to encompass proposed genus Zybavirus by Dépieu et al. (2016)). For each genus-level taxon, the number of characterized genome segments for each virus (1 or 2) and known hosts (P, plants; F, fungi; A, alveolate protist) are indicated.
including only 1 position (a Gly residue) that is wholly conserved among the 22 ORF1p sequences included in this comparison. The ORF1p alignment nevertheless appears robust in including gaps at only 4 alignment positions besides in the terminal regions and in having 89 alignment positions (22%) at which least similar residues are found in all 22 of the sequences (Fig. S2). A large central portion of this alignment is nearly identical with one generated using PROMALS3D, which additionally predicts a consensus secondary structure comprising many α-helices and notably no β-strands (Fig. S2). Prediction of predominantly α-helical content for amalgavirus ORF1p has been previously reported (Sabanadzovic et al., 2009, 2010; Kruppovic et al., 2015). In addition, we newly observed that a central span of 19–46 residues is predicted in all of the different proposed and approved plant amalgaviruses to form an α-helical coiled coil structure (Fig. S2, Fig. 4B), which would be an unusual finding for a viral CP that assembles into an icosahedral particle. This new observation may thus support the suggestion that amalgavirus ORF1p forms some other type of structure, such as a filamentous nucleocapsid (Krupovic et al., 2015) or a more amorphous replication factory matrix (Ishigai et al., 2011). Interestingly, too, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but surprisingly, too, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTTV-like viruses (all but unexpectedly, the ORF1 products from ZbV-Z and unirnaviruses, as well as the R...
backward by one codon to CUU_AGU_C in CaAV1 and still allowing P-site anticodon-codon pairing after ribosomal slippage from CUU to UUA (Fig. 1C).

Interestingly, the same heptanucleotide, CUU_AGU_C, is utilized for highly efficient +1 PRF in Saccharomyces cerevisiae Ty1, Ty2, and Ty4 elements (Belcourt and Farabaugh, 1990). There, high efficiencies (up to ~40%) depend in part on the low availability in S. cerevisiae of the tRNAArg with anticodon 3’-UCC. In plants, however, this tRNA appears not to be limiting so that frameshifting efficiencies may be much lower, perhaps consistent with the ~1–2% frameshifting efficiencies measured in rabbit reticulocyte lysates for the UUU_CGN influenza A virus shift site seemingly shared by other amalgaviruses (Jagger et al., 2012). Notably, the codon proposed to be in the A site at the onset of frameshifting differs between CaAV1 (AGU, encoding Ser) and STV (AGG, encoding Arg). Similarly, for the sequences with proposed UUU_CGN shift sites, all four CGN arginine codons (corresponding to three tRNAArg iso-acceptors) are represented. This suggests there may be specific features of CGN and AGN A-site codons, other than simply the availability of the cognate tRNA (and aside from the obvious restriction at the first codon position, C or A, to permit +1 repairing of the P-site tRNA), that favor P-site +1 slippage.

UvNV1 and NoURV1 (Zhang et al., 2014; Zhou et al., 2016) (see Table S2 for abbreviations and GenBank numbers) are two recently described mycoviruses with monosegmented dsRNA genomes that have ORF2 (encoding RdRp) positioned in the +1 frame relative to ORF1. They are related to each other but, according to phylogenetic analyses with RdRp sequences, they are more distantly related to plant amalgaviruses than is amalga-like mycovirus ZbV-Z (e.g., see Fig. 3). Notably, however, both UvNV1 (Zhang et al., 2014) and NoURV1 (this report) have motif UUU_CGA properly positioned in the region of ORF1–ORF2 overlap to be their potential +1 PRF site. Also, the ORF1 translation product of each, which is quite small (172 or 174 aa), is predicted to be predominantly α-helical in secondary structure and to have propensity for coiled coil formation (Fig. S4). Primary sequence conservation across the ORF1 products of plant amalgaviruses, ZbV-Z, and UvNV1 and NoURV1 appears limited. However, with MAFFT (Fig. S2) as well as several other alignment programs, we noted a 100–150 aa central region of ORF1p from all these viruses that aligned in three large blocks with no gaps, including across the largely conserved Gly residue and the region with consistently predicted coiled coil propensity (Fig. S2). These findings suggest to us that ORF1p from plant amalgaviruses, ZbV-Z, and UvNV1 and NoURV1 are indeed all homologs, thus presumably sharing a common ancestor.

In our original tblastn search against the TSA database for plants, we found a number of additional accessions with E-value scores between 0.0 and 1e–30, indicative of still strong similarities with the BLV ORF1+2p query. Fourteen of these accessions were from 9 different plant species not represented in Table 1 (Agropyron cristatum, Atractylodes lancea, Camellia sinensis, Frigillaria ciliaris, Gentiana macrophylla, Phalaenopsis aphrodite, Prosopis alba, Reaumuria trigyna, and Solanum melongena); however, none of them were >1898 nt in length (Table S2), such that they do not approach the genome lengths of plant amalgaviruses. When used in a subsequent blastx search against the full NR database, each of these 14 TSA accessions scored most highly nonetheless with one of the four originally characterized plant amalgaviruses (E-value scores ≤8e–32). Moreover, upon examining their sequences, we found that one reading frame of each accession approximates an end-to-end ORF, the translated product of which in a PSI-BLAST search showed protein sequence similarity across approximately its full length with at least one of the original amalgaviruses (E-value scores ≤4e–38). We therefore consider it likely that the TSA accessions listed in Table S3 represent partially determined sequences of yet other bona fide amalgaviruses, which were infecting these additional plant species at the times of sampling for transcriptome analyses. TSA accessions with E-value scores >1e–30 in the initial tblastn search may also hold interesting findings but were outside the focus of this study.

The TSA accessions and SRA data sets used in this study are associated with peer-reviewed publications in some cases (Czaban et al., 2015; Duangjit et al., 2013; Farrell et al., 2014; Gould et al., 2015; Khalil et al., 2015), but not in others. Moreover, none of the TSA accessions are currently annotated to indicate their viral origins. This lack of annotation will make it difficult for many investigators to locate these sequences for inclusion in phylogenetic analyses or other comparisons. We have therefore been attempting, though without success to date, to deposit the newly proposed plant amalgavirus sequences summarized in Table 1 as Third-Party Annotations at GenBank, in an effort to make them easier to locate via their metadata. A more routine procedure for encouraging and accepting such new deposits based on sequence data previously made public at NCBI – especially those sequence data in the TSA, SRA, and other databases that have been rapidly expanding consequent to next-generation sequencing methods – seems likely to be of broad benefit.

4. Materials and methods

All database searches were performed with the indicated programs as implemented with defaults at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Searches of the TSA database with protein sequence queries deduced from nucleotide sequences were performed using blastx. Searches of the SRA database with nucleotide sequence queries were performed using discontiguous megablast. For the TSA and SRA searches, default settings were sometimes altered to allow larger numbers of target sequences (>100) to be displayed. Searches of the NR database with nucleotide sequence queries or with protein sequence queries deduced from nucleotide sequences were performed using blastx or PSI-BLAST, respectively.

Given the incomplete protein-coding regions in some of the amalgavirus-like TSA accessions that we first discovered (GAMH01005363, GAYX01076418, GBIE01028534, GBXZ01099138, GCJW01039808, GEAC01063629, and GEC001025317; Table 1, top), we accessed the TSA data sets from each of those transcriptome projects and in discontiguous megablast searches found reads that mapped to each of the original TSA accessions. We then used CAP3 (Huang and Madan, 1999) or CLC Genomics Workbench 8 (Qiagen) to assemble contigs that were compared with the TSA sequence. In the cases of TSA accessions GAYX01076418, GBXZ01099138, GCJW01039808, GEAC01063629, and GEC001025317, we were able to extend the original sequence at one or both termini in this manner. We reiteratively repeated this process to add new SRA accessions to each extending terminus until newly matching accessions were no longer found. The SRA data sets searched for each of the originally truncated TSA sequences were: GAMH01005363, SRX329048 and SRX329051; GAYX01076418, SRX670823–SRX670828; GBIE01028534, SRX1733822–SRX1733825; GBXZ01099138, SRX757593; GCJW01039808, DRX000652–DRX000659; GEC001063629, SRX1374921–SRX1374944; and GEC001025317, SRX1427125–SRX1427157.

ORFs were identified in nucleotide sequences using EMBOSSEXORF as implemented at http://www.bioinformatics.nl/emboss-explore/ or ExPaSY Translate as implemented at http://web.expasy.org/translate/. Multiple sequence alignments of RNA or protein sequences were performed using MAFFT 7.2 (L-INS-i) (Katoh and Standley, 2013) as implemented with defaults at http://mafft.cbrc.jp/alignment/server/. Multiple sequence alignments accompanied by secondary structure predictions were obtained using PROMALS3D (Petti and Grishin, 2014) as implemented with defaults.
at http://prodata.swmed.edu/promals3d/promals3d.php. Global pairwise alignments of protein sequences were performed using Needle ( Needleman and Wunsch, 1970 ) or Needleall as implemented with defaults at http://www.bioinformatics.nl/emboss-explorer/. Average degree of conservation along a multiple sequence alignment was plotted using EMBOSS: plot as implemented with defaults (except window size = 10) at http://www.bioinformatics.nl/emboss-explorer/. Coiled coil predictions were obtained using MARCOIL or COILS/PCoisls ( Lupas, 1996 ) as implemented with defaults at http://toolkit.tuebingen.mpg.de/.

Phylogenetic relationships were determined using PhyML 3.0 ( Guindon et al., 2010 ) as implemented at http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html with the following parameters differing from the defaults: Sequence type/model, Amino acids/LG or rtREV; Proportion of invariable sites, estimated from data; Gamma shape parameter, estimated from data; Starting tree ( s ) optimization, Tree topology and Branch length; Tree improvement, Best of NNI and SPR; Branch support, Approximate Likelihood Ratio Test ( aLRT ), SH-like supports. The results in Newick format were then submitted to TreeDyn 198.3 as implemented at http://www.phylogeny.fr/ for displaying branch support values in % and collapsing branches with lower support values. The output in Newick format was then opened in FigTree v1.4.0 (downloaded from http://tree.bio.ed.ac.uk/software/figtree/) for refining the phylogram for presentation.

Table S2 lists abbreviations and GenBank accession numbers for nucleotide sequences of other dsRNA viruses included in this study besides those in Table 1 and Table S1. The ORF2p ( RdRp ) sequences of other dsRNA viruses included in this study

References


Appendix A. Supplementary material

Supplementary data can be found with this article in the online version at http://dx.doi.org/10.1016/j.virol.2016.07.002.

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References


