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Accessibility
A +1 ribosomal frameshifting motif prevalent among plant amalgaviruses

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Abstract

Sequence accessions attributable to novel plant amalgaviruses have been found in the Transcriptome Shotgun Assembly database. Sixteen accessions, derived from 12 different plant species, appear to encompass the complete protein-coding regions of the proposed amalgaviruses, which would substantially expand the size of genus Amalgavirus from 4 current species. Other findings include evidence for UUU_CGN as a +1 ribosomal frameshifting motif prevalent among plant amalgaviruses; for a variant version of this motif found thus far in only two amalgaviruses from solanaceous plants; for a region of α-helical coiled coil propensity conserved in a central region of the ORF1 translation product of plant amalgaviruses; and for conserved sequences in a C-terminal region of the ORF2 translation product (RNA-dependent RNA polymerase) of plant amalgaviruses, seemingly beyond the region of conserved polymerase motifs. These results additionally illustrate the value of mining the TSA database and others for novel viral sequences for comparative analyses.

Keywords:
Amalgaviridae
Coiled coil
Database mining
dsRNA virus
Fungal virus
Plant virus
Ribosomal frameshifting

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 virions. 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 (Isgai 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/DBJ 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/DBJ 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...
between 2793 and 3478 nt, approximating the genome lengths of previously characterized plant amalgaviruses (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 amalgaviruses 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 amalgaviruses? Strikingly, as in previously characterized plant amalgaviruses, 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 amalgaviruses. 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 amalgaviruses (Firth et al., 2012) and the amalga-like mycovirus Zygosaccharomyces 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 one or more stop codon) and encodes a smaller-than-expected protein product (Table 1, top). These 7 sequences hence appear to be truncated at one or both ends, the lengths are calculated to the termini and are listed in parentheses.

### Table 1

<table>
<thead>
<tr>
<th>Putative host species (cultivar)</th>
<th>GenBank accession no.</th>
<th>Amalgavirus (abbrev.)</th>
<th>Length (bp)a</th>
<th>ORF1p (aa)b</th>
<th>ORF2p (aa)c</th>
<th>ORF1 + 2p (aa)d</th>
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</thead>
<tbody>
<tr>
<td><em>Allium cepa</em> (OH1)</td>
<td>GAAC001100618*</td>
<td>AcAV1</td>
<td>3453</td>
<td>391</td>
<td>779</td>
<td>1057</td>
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<td>AcAV2</td>
<td>3453</td>
<td>390</td>
<td>781</td>
<td>1065</td>
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<tr>
<td><em>Anthoxanthum odoratum</em></td>
<td>GBIE01024896*</td>
<td>AoAV1</td>
<td>3356</td>
<td>382</td>
<td>783</td>
<td>1056</td>
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<tr>
<td><em>Anthoxanthum odoratum</em></td>
<td>GBIE01028534*</td>
<td>AoAV2</td>
<td>(2971)</td>
<td>(388)</td>
<td>(716)</td>
<td>(989)</td>
</tr>
<tr>
<td><em>Camellia oleifera</em> (Xianglin4)</td>
<td>GEFY0004381</td>
<td>CoAV1</td>
<td>3333</td>
<td>398</td>
<td>774</td>
<td>1066</td>
</tr>
<tr>
<td><em>Capsicum annuum</em> (CM334)</td>
<td>JW101175</td>
<td>CaAV1</td>
<td>3478</td>
<td>375</td>
<td>774</td>
<td>1062</td>
</tr>
<tr>
<td><em>Clovea drosserifolia</em></td>
<td>GDRE01029069*</td>
<td>CdAV1</td>
<td>3443</td>
<td>402</td>
<td>774</td>
<td>1070</td>
</tr>
<tr>
<td><em>Ergirven breviscapuc</em></td>
<td>GDQF01098448</td>
<td>EbAV1</td>
<td>3433</td>
<td>384</td>
<td>784</td>
<td>1049</td>
</tr>
<tr>
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<td>GDQF01120453</td>
<td>EbAV2</td>
<td>3408</td>
<td>386</td>
<td>785</td>
<td>1052</td>
</tr>
<tr>
<td><em>Festuca pratensis</em> (Laura)</td>
<td>GBXZ01002308*</td>
<td>FpAV1</td>
<td>3411</td>
<td>385</td>
<td>774</td>
<td>1053</td>
</tr>
<tr>
<td><em>Festuca pratensis</em> (Laura)</td>
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<td>FpAV3</td>
<td>(3288)</td>
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<td>(768)</td>
<td>(1047)</td>
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<td><em>Gevania avellana</em> (Mol.)</td>
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<td>(2793)</td>
<td>(228)</td>
<td>774</td>
<td>(896)</td>
</tr>
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<td><em>Lolium perenne</em> (P226/135/16)</td>
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<td>LpAV1</td>
<td>3296</td>
<td>385</td>
<td>(770)</td>
<td>(1049)</td>
</tr>
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<td>MsAV1</td>
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<td>772</td>
<td>1058</td>
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<td>PeAV1</td>
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<td>1059</td>
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<td>PsAV1</td>
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<td>(322)</td>
<td>777</td>
<td>(1003)</td>
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<td><em>Salicornia europaea</em></td>
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<td>ScAV1</td>
<td>(2798)</td>
<td>382</td>
<td>(613)</td>
<td>(880)</td>
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<td>ScAV1</td>
<td>(2851)</td>
<td>382</td>
<td>(633)</td>
<td>(916)</td>
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<td>3431</td>
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<td>774</td>
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<td><em>Rhododendron virus A</em></td>
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<td>RVH-A</td>
<td>3427</td>
<td>404</td>
<td>777</td>
<td>1077</td>
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<td><em>Southern tomato virus</em></td>
<td>EF442780</td>
<td>STV</td>
<td>3437</td>
<td>377</td>
<td>774</td>
<td>1062</td>
</tr>
<tr>
<td><em>Vicia clypturn</em></td>
<td>EU317895*</td>
<td>VCV-M</td>
<td>3434</td>
<td>394</td>
<td>771</td>
<td>1057</td>
</tr>
</tbody>
</table>

---

* Nucleotide 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 in-frame Met residue 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.
* For apparently full-length ORF2 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 first in-frame stop codon in ORF2p, taking into account the proposed +1 PRF site. For ORF1 + 2 translation products that appear to be truncated at one or both ends, the lengths are calculated 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).
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, RFlV-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 tRNA^Phe iso-acceptor that has anticodon 3′AAG (Grojean et al., 2010). First positioned on codon UUU in the +1 PRF motif, this tRNA is thought to slip forward by one nucleotide (arrow) in the P site (onto codon UUC), positioning the next codon (AGG) in the A site for continued translation. (B) Previously proposed motif from plant amalgavirus STV (Depierreux et al., 2016) is shown. Anticodon 3′AAG is thought to slip forward by one nucleotide in the P site (onto codon GGC), positioning the next codon (GNN) in the A site for continued translation. (C) Newly proposed motif from plant amalgavirus 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 GGC), positioning the next codon (GCC) 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/Solanoidaeae; MsAV1 and VCV-M, Fabaceae/Faboidaeae; AoAV1 and FpAV1, Poaceae/Pooideae; and FpAV3 and LpAV1, Poaceae/Pooideae. 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 CTVV, which are presumably all bisegmented (Batella 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 rKev 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 equivalently u, tiny). There are also regions of strong sequence conservation (h, hydrophobic; n, negatively charged; p, polar; s, small; t, turn-like; c, 445-CAGDDNLT-454). 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 equivalently u, tiny). There are also regions of strong sequence conservation (h, hydrophobic; n, negatively charged; p, polar; s, small; t, turn-like; c, 445-CAGDDNLT-454).
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 Depierreux 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 at 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; Krupovic 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 (Isoigai et al., 2011). Interestingly, too, the ORF1 products from ZbV-Z and unirnaviruses, as well as the RNA2 products from most CTV-like viruses (but all RHsDRV1; see Table S2 for abbreviations and GenBank numbers), are also predicted to form α-helical coiled coil structures (Fig. S4), suggesting that the non-RdRp proteins from all these clades may share structural and functional characteristics, and possibly a common ancestor. See Discussion for additional considerations in this regard.

The two TSA accessions from A. cepa (bulb onion), which we now propose to represent novel plant amalgaviruses (Table 1), were derived respectively from two cultivars, OH1 and DHS5225, seeds of which were gifted to us by Dr. Michael J. Havey (USDA-ARS and University of Wisconsin-Madison). Using internal primers designed from these two accessions, we were able to generate RT-PCR amplicons of expected sizes (825–875 bp) from RNA isolated from shoots (OH1) or seeds (DHS5225) of these two cultivars. Moreover, upon Sanger sequencing of the amplicons, we found their sequences to be ≥ 99.5% identical to those of the respective TSA accessions (matching nt 1710–2531 of OH1 and nt 1522–2313 of DHS5225). These findings provide further evidence that each of these two A. cepa cultivars is persistently infected with the respective amalgavirus.

3. Discussion

One question that arises is whether the TSA-derived sequences characterized here (see Table 1) represent transcripts of chromosomal or extrachromosomal, host or viral, origin. In recent years, remnants of many nonretroviral RNA virus genomes have been found integrated in host chromosomes (Chiba et al., 2011; Katzourakis and Gifford, 2010; Taylor and Bruehn, 2009) and, if transcribed, may be detected in transcript-derived databases. In the vast majority of these cases, however, the integrated viral elements are notably fragmented, and their ORFs are disrupted by stop codons and frame-shift mutations. This is not unlike the case for the TSA-derived sequences listed in Table 1, which approximate the lengths of complete plant amalgavirus genomes and have the expected long ORFs for expressing ORF1p and ORF1+2p. Thus, we conclude that all of the TSA accessions in Table 1 likely represent bona fide plant amalgaviruses, which were infecting the respective plants at the times of sampling for transcriptome analyses.

The TSA accession from C. annuum, representing putative amalgavirus CaAV1, is notable for lacking a copy of the UUU_CGN consensus motif for +1 PRF in its ORF1–ORF2 overlap region. As noted above, CaAV1 is quite similar to STV in pairwise comparisons (Fig. 2), and indeed their two RdRp sequences approach an identity threshold (65–70%) often used for assigning RNA virus strains to the same or different species. Interestingly, STV is also like CaAV1 in lacking a copy of the UUU_CGN consensus motif for +1 PRF in its ORF1–ORF2 overlap region (Depierreux et al., 2016; Firth et al., 2012), and their respective plants of origin, tomato and pepper, are members of the same taxonomic family and subfamily, Solanaceae/Solanoidae, indeed of two closely related tribes, Solanaceae and Capsicaceae, within that subfamily (Särkinnen et al., 2013).

In an effort to identify an atypical +1 PRF motif in CaAV1, we examined the multiple sequence alignments of both the plus-strand RNA and the full-length ORF2 translation products of the proposed and original plant amalgaviruses (Fig. S3). Based on these alignments, the motif for +1 PRF in CaAV1 is predicted to be CUU_AGU_C (Fig. 1C), where translation of the CUU codon is followed by translation of the GUC codon consequent to +1 PRF.

Notably with this motif, the anticodon 3′-GAI (1=inosine) decoding codon CUU (Grojean et al., 2010) could remain engaged in the ribosomal P site upon forward slippage to codon UUA, including a G:U pair in the first position. Although the +1 shift in STV was previously suggested to occur on motif AGG_CGU_C (see Fig. 1B), based on the RNA alignment (Fig. S3) and other considerations, we now suggest that the +1 PRF motif of STV would be better revised...
backward by one codon to CUU\_AGG\_C, making it very similar to CUU\_AGG\_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\_AGG\_C, is utilized for highly efficient +1 PRF in Saccharomyces cerevisiae Ty1, Ty2, and Ty4 elements (Belcourt and Farabaugh, 1990). There, high S. cerevisiae have ORF2 (encoding RdRp) positioned in the described mycoviruses with monosegmented dsRNA genomes that lized for highly ef

likely that the TSA accessions listed in Table S3 represent partially across approximately its full length with at least one of the original homologies with the BLV ORF1 appears to be predominantly α-helical in secondary structure and to have propensity for coiled coil forma-

Given the incomplete protein-coding regions in some of the amalgavirus-like TSA accessions that we first discovered (GAMH01005363, GAYX01076418, GBIE01028534, GBXZ01009138, GCJW01039808, GEAC01063629, and GECO01025317; Table 1, top), we accessed the TSA data sets from each of those transcriptome projects and in contiguous megablast searches found reads that mapped to each of the original TSA sequences. 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, GBXZ01009138, GCJW01039808, GEAC01063629, and GECO01025317, 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; GBXZ01009138, SRX757539; GCJW01039808, DRX000652–DRX000659; GECO01063629, SRX1374921–SRX1374944; and GECO01025317, SRX1427152–SRX1427157.

ORFs were identified in nucleotide sequences using EMBOSS getorf as implemented at http://www.bioinformatics.nl/emboss-explorer/ 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 (Pei and Grishin, 2014) as implemented with defaults

4. Materials and methods

All database searches were performed with the indicated pro-

grams 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 tblastn. Searches of the SRA database with nucleotide sequence queries were performed using discontinuous 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, GBXZ01009138, GCJW01039808, GEAC01063629, and GECO01025317; Table 1, top), we accessed the TSA data sets from each of those transcriptome projects and in contiguous megablast searches found reads that mapped to each of the original TSA sequences. 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, GBXZ01009138, GCJW01039808, GEAC01063629, and GECO01025317, 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; GBXZ01009138, SRX757539; GCJW01039808, DRX000652–DRX000659; GECO01063629, SRX1374921–SRX1374944; and GECO01025317, SRX1427152–SRX1427157.

ORFs were identified in nucleotide sequences using EMBOSS getorf as implemented at http://www.bioinformatics.nl/emboss-explorer/ 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 (Pei and Grishin, 2014) as implemented with defaults
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/PCOILS (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/interactive.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 Newick format were then submitted to TreeDyn 198.3 as implemented at http://www.phylogeny.fr/ for displaying branch support values in

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 were retrieved from http://tree.bio.ed.ac.uk/software/figtree/ for refining the phylogram for presentation.

References


