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Genome Sequences of Spinach Deltapartitivirus 1, Spinach Amalgavirus 1, and Spinach Latent Virus Identified in Spinach Transcriptome

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Complete genome sequences of three new plant RNA viruses, Spinach deltapartitivirus 1 (SpDPV1), Spinach amalgavirus 1 (SpAV1), and Spinach latent virus (SpLV), were identified from a spinach (*Spinacia oleracea*) transcriptome dataset. The RNA-dependent RNA polymerases (RdRps) of SpDPV1, SpAV1, and SpLV showed 72%, 53%, and 93% amino acid sequence identities with the homologous RdRp of the most closely related virus, respectively, suggesting that SpDPV1 and SpAV1 were novel viruses. Sequence similarity and phylogenetic analyses revealed that SpDPV1 belonged to the genus *Deltapartitivirus* of the family *Partitiviridae*, SpAV1 to the genus *Amalgavirus* of the family *Amalgaviridae*, and SpLV to the genus *Ilarvirus* of the family *Bromoviridae*. Based on the demarcation criteria, SpDPV1 and SpAV1 are considered as novel species of the genera *Deltapartitivirus* and *Amalgavirus*, respectively. This is the first report of these two viruses from spinach.

Keywords: Spinach deltapartitivirus 1, Spinach amalgavirus 1, Spinach latent virus, spinach, transcriptome

Introduction

Spinach (*Spinacia oleracea*) is an annual plant of the family Amaranthaceae that is widely cultivated as an economically important leafy vegetable crop. In 2014, the world total production of spinach was 24.3 million tons, about 92% of which was produced in China and the USA according to the Food and Agriculture Organization of the United Nations (http://www.fao.org). Breeding programs and genetic studies of spinach have investigated the development of varieties with desirable traits such as increased disease resistance and stress tolerance [1, 2]. In addition, comprehensive genomic and transcriptomic studies of spinach have been actively performed to evaluate the genetic diversity of spinach and identify molecular resources, including a comparison of spinach sex chromosomes with sugar beet autosomes [3], a comparative transcriptomic analysis of cultivated and wild spinach varieties [4], and an expression profiling study of spinach leaves under heat stress [5].

A wide range of RNA viruses infect plants [6]. Plant RNA viruses have four different lifestyles: acute, chronic, endogenous, and persistent [7]. Persistent viruses have been often ignored because they are generally asymptomatic and exist at a low titer. Currently, at least 18 viruses, including 14 RNA viruses, are known to infect spinach: these 14 RNA viruses are Beet black scorch virus, Beet chlorosis virus, Beet mild yellowing virus, Beet mosaic virus, Beet necrotic yellow vein virus, Beet western yellows virus, Broad bean wilt virus 1, Lettuce mosaic virus, Melon yellow spot virus, Pepper ringspot virus, Spinach cryptic virus 1, Spinach latent virus (SpLV), Tobacco rattle virus, and Turnip yellows virus (http://www.genome.jp/virushostdb/3562; last accessed May 2, 2017) [8]. Most of these viral infections are acute or chronic and produce visible symptoms. Information about persistent, non-symptomatic viral infections of spinach is highly limited. The identification of novel viruses that infect spinach may help to prevent compromise of the quality and yield of spinach.

Next-generation sequencing-based RNA-Seq studies have been widely performed for the transcriptomic analysis of various plants. The genomic RNA fragments of plant RNA viruses can be isolated during RNA preparation together with the host RNA molecules, and their sequences can be identified from RNA-Seq datasets and the Transcriptome

Shotgun Assembly (TSA) database [9-12].

Previously, we identified Spinach cryptic virus 1, a novel member of the genus *Alphapartitivirus* [13], from a spinach transcriptome dataset (SRP052590) [4]. In this study, we analyzed another spinach transcriptome dataset (SRP059420) and identified three new viruses that are members of the genera *Deltapartitivirus*, *Amalgavirus*, and *Ilarvirus*, respectively.

Materials and Methods

Spinach (*Spinacia oleracea*) transcriptome data obtained from root samples were downloaded from the Sequence Read Archive of the National Center for Biological Information (NCBI). The dataset with accession number SRP059420 contained 46.6 Gbp of RNA-Seq data (https://www.ncbi.nlm.nih.gov/sra/SRP059420). The data were quality controlled using Sickle (ver. 1.33; available at https://github.com/najoshi/sickle) with the parameters "-q 30 -l 50." The whole trimmed RNA-Seq reads were assembled into contigs using the SPAdes Genome Assembler (ver. 3.10) [14].

The genome sequences of dsRNA viruses (NCBI txid 35325) and ssRNA viruses (txid 439488) of more than 500 bp in length were downloaded from the NCBI Nucleotide database and converted into a BLAST-searchable database. A BLASTN search was conducted to identify possible viral RNA sequences in the assembled contigs [15]. The E-value cut-off was 1e−5. Contigs with an aligned length of 300 bp or longer were subjected to BLASTN or BLASTX searches against the non-redundant nucleotide or protein database, respectively, at the NCBI BLAST Web site (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The contigs with full-length viral protein-coding regions were collected and annotated.

To investigate the heterogeneity of the assembled viral genome sequences, the RNA-Seq reads were mapped back to the corresponding contigs using the Burrows-Wheeler alignment tool with the default parameters [16]. Sequence variations were identified using SAMtools and BCFtools [17]. The Integrative Genomics Viewer software program was used to examine sequence variations and sequencing depths [18].

For the phylogenetic analysis, homologous RNA-dependent RNA polymerase (RdRp) sequences were collected and multiply aligned using MUSCLE [19]. Phylogenetic trees were inferred using the neighbor-joining method implemented in the MEGA7 software [20]. The prediction of protein secondary structure was conducted using PSIPRED (ver. 3.3) (http://bioinf.cs.ucl.ac.uk/psipred/) [21]. A sequence logo was generated using WebLogo 3 (http://weblogo.threeplusone.com) [22, 23].

Results and Discussion

We identified three viruses that may be associated with spinach by analyzing the spinach root transcriptome dataset SRP059420 (Table 1). Sequence similarity searches and phylogenetic analyses revealed that two of the identified viruses were novel species of the genera *Deltapartitivirus* and *Amalgavirus*, respectively. The third identified virus showed similarity to a previously reported SpLV of the genus *Ilarvirus*.

Spinach Deltapartitivirus 1 (SpDPV1)

Among the three spinach viruses identified in this study, one virus consisted of two sequence contigs that showed similarities to the RNA1 and RNA2 segments, respectively, of the viruses belonging to the genus *Deltapartitivirus* of the family *Partitiviridae*, including Fig cryptic virus (FCV) [24]. Therefore, these two contigs were considered as the genomic segments of a putative member of the genus *Deltapartitivirus*, which was named as SpDPV1.

The SpDPV1 RNA1 sequence was 1,683 nucleotides (nt) in length and encoded an RdRp comprising 471 amino

Table 1. Summary of the spinach RNA viruses identified in this study.

Full name	Abbrev.	Segment	Accession	Length (nt)	ORF	Position	Length (aa)
Spinach deltapartitivirus 1	SpDPV1	RNA1	KY695009	1,683	RNA-dependent RNA polymerase[a]	132–1,547	471
		RNA2	KY695010	1,475	Coat protein	223–1,320	335
Spinach amalgavirus 1	SpAV1	RNA1	KY695011	3,420	ORF1p (putative coat protein)	146–1,309	387
					ORF1+2p (fusion protein)[a]	146–955, 957–3,308	1,053
Spinach latent virus	SpLV	RNA1	KY695012	3,425	Replicase	63–3,233	1,056
		RNA2	KY695013	2,931	Polymerase[a]	65–2,458	797
					2b protein	2,215–2,738	195
		RNA3	KY695014	2,295	Movement protein	350–1,198	282
					Coat protein	1,329–1,985	218

[a]Proteins containing the RNA-dependent RNA polymerase motif.

acids (aa) (Table 1). The RNA2 segment was 1,475 nt in length and encoded a 335-aa coat protein (CP). Two single nucleotide polymorphism sites, one in each of the two segments, were identified from the analysis of the RNA-Seq reads. One nucleotide polymorphism (A/G) was found at the base position 156 in the RNA1 segment, which resulted in a threonine/alanine aa polymorphism. The other nucleotide polymorphism (G/C) was located at position 201 in the RNA2 segment and was within the 5′-untranslated region (UTR) of the CP coding region.

The SpDPV1 RdRp encoded by the RNA1 segment showed a 72% aa sequence identity with the RdRp of FCV, which is the most closely related known virus (Table 2). The SpDPV1 RdRp also showed a 35–40% aa sequence identity with other viruses of the genus *Deltapartitivirus* or unclassified genera in the family *Partitiviridae*. These viruses included Raphanus sativus cryptic virus 2 (RsCV2), Arhar cryptic virus-I (ArCV-I), Fragaria chiloensis cryptic virus (FcCV), Rose cryptic virus 1 (RoCV1), and Rosa multiflora cryptic virus (RmCV). Multiple sequence alignment of the SpDPV1 RdRp and its homologs from related plant-infecting viruses revealed the eight conserved motifs regarded as a hallmark of the *Partitiviridae* RdRps [25]. The demarcation criteria for the genus *Deltapartitivirus* is that aa sequence identities among RdRps of deltapartitiviruses range 33.5–87.7% [26]. Therefore, we concluded that SpDPV1 is a novel species of the genus *Deltapartitivirus* from the family *Partitiviridae*. This is the first report of *Deltapartitivirus* species identified in spinach.

The SpDPV1 CP encoded by the RNA2 segment showed a 60% aa sequence identity with the CP of the most closely related virus (FCV). None of the other *Deltapartitivirus* CPs showed a significant identity with the SpDPV1 CP, indicating that CPs generally evolve faster than RdRps [27].

A phylogenetic analysis using the SpDPV1 RdRp and its related sequences also confirmed that SpDPV1 is a novel species of the genus *Deltapartitivirus* (Fig. 1A). SpDPV1 showed the closest relationship with FCV and clustered in a clade comprising five other deltapartitiviruses (RsCV2, ArCV-I, FcCV, RoCV1, and RmCV). The host plants of these seven *Deltapartitivirus* species can be classified into four different orders of eudicots: Caryophyllales (spinach), Rosales (fig, beach strawberry, multiflora rose, and rose), Brassicales (radish), and Fabales (pigeon pea). Interestingly, spinach (the host of SpDPV1) and fig (the host of FCV) belong to the clades superasterid and superrosid, respectively, which diverged more than 100 million years ago [28]. The wide range of host plants and the discordance of phylogenetic tree topologies between viruses and their hosts strongly

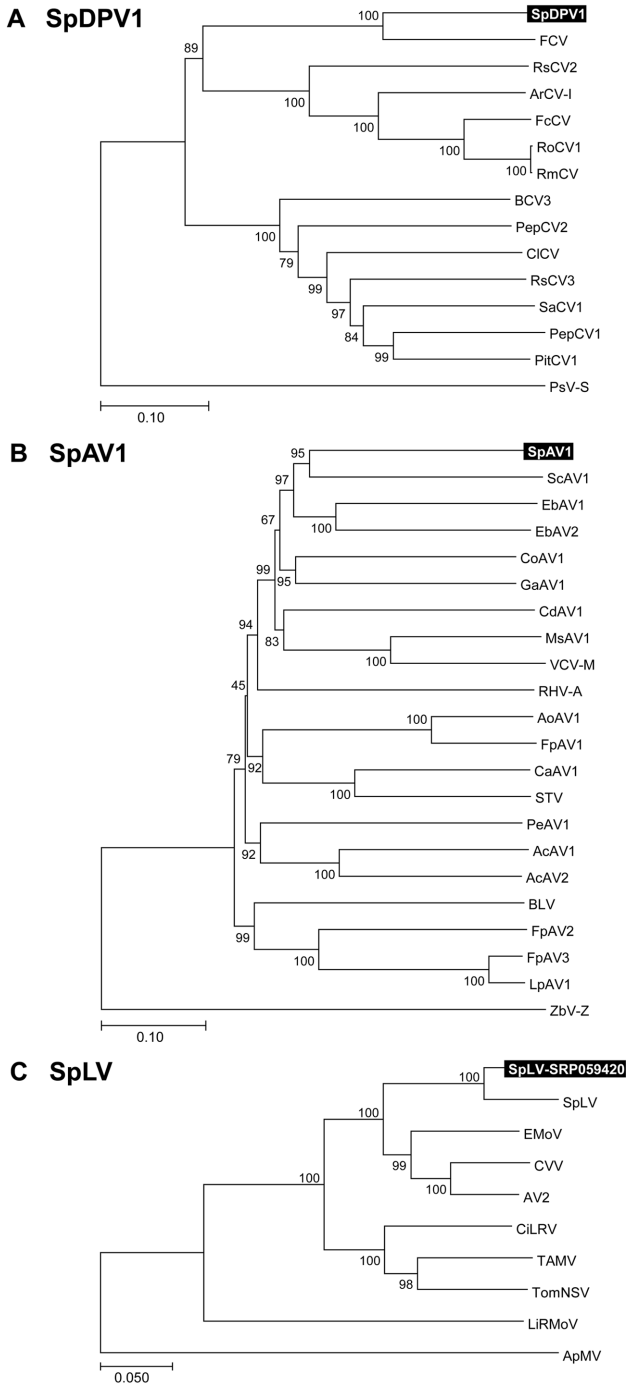

Fig. 1. Phylogenetic analysis of newly identified spinach RNA viruses.

RNA-dependent RNA polymerase motif-containing proteins of SpDPV1 (**A**), SpAV1 (**B**), and SpLV (**C**) were aligned with their respective homologs of the genera *Deltapartitivirus*, *Amalgavirus*, and *Ilarvirus*. PsV-S, ZbV-Z, and ApMV were separately used as outgroups. The bootstrap percentages calculated from 1,000 bootstrap replicates are shown at the nodes. See Table 2 for the full names and accession numbers of the viruses.

Table 2. Identities among the RdRp sequences of SpDPV1, SpAV1, SpLV, and their respective related viruses.

	Full name	Abbrev.	Accession[a]	Amino acid identity[b]
SpDPV1	Fig cryptic virus	FCV	NC_015494.1	340/469 (72%)
	Raphanus sativus cryptic virus 2	RsCV2	NC_010343.1	183/460 (40%)
	Arhar cryptic virus-I	ArCV-I	NC_024014.1	183/470 (39%)
	Fragaria chiloensis cryptic virus	FcCV	NC_009519.1	185/470 (39%)
	Rose cryptic virus 1	RoCV1	NC_010346.1	185/470 (39%)
	Rosa multiflora cryptic virus	RmCV	EU024675.1	186/470 (40%)
	Beet cryptic virus 3	BCV3	S63913.1	182/454 (40%)
	Pepper cryptic virus 2	PepCV2	LC195294.1	172/476 (36%)
	Citrullus lanatus cryptic virus	ClCV	KY081285.1	174/462 (38%)
	Raphanus sativus cryptic virus 3	RsCV3	NC_011705.1	174/453 (38%)
	Sinapis alba cryptic virus 1	SaCV1	NC_030243.1	164/435 (38%)
	Pepper cryptic virus 1	PepCV1	JN117276.1	171/454 (38%)
	Pittosporum cryptic virus-1	PitCV1	LN680393.2	165/453 (36%)
	Penicillium stoloniferum virus S	PsV-S	NC_005976.2	77/334 (23%)
SpAV1	Secale cereale amalgavirus 1	ScAV1	GCJW01039808.1	545/1,031 (53%)
	Erigeron breviscapus amalgavirus 1	EbAV1	GDQF01098448.1	524/1,035 (51%)
	Erigeron breviscapus amalgavirus 2	EbAV2	GDQF01120453.1	526/1,038 (51%)
	Camellia oleifera amalgavirus 1	CoAV1	GEFY01004381.1	502/1,043 (48%)
	Gevuina avellana amalgavirus 1	GaAV1	GEAC01063629.1	516/1,035 (50%)
	Cleome droserifolia amalgavirus 1	CdAV1	GDRJ01026949.1	490/1,060 (46%)
	Medicago sativa amalgavirus 1	MsAV1	GAFF01077243.1	473/1,035 (46%)
	Vicia cryptic virus M	VCV-M	EU371896.1	461/1,045 (44%)
	Rhododendron virus A	RHV-A	NC_014481.1	397/706 (56%)
	Anthoxanthum odoratum amalgavirus 1	AoAV1	GBIE01024896.1	430/1,019 (42%)
	Festuca pratensis amalgavirus 1	FpAV1	GBXZ01049574.1	419/1,002 (42%)
	Capsicum annuum amalgavirus 1	CaAV1	JW101175.1	436/1,066 (41%)
	Southern tomato virus	STV	NC_011591.1	429/1,067 (40%)
	Phalaenopsis equestris amalgavirus 1	PeAV1	GDHJ01028335.1	439/1,002 (44%)
	Allium cepa amalgavirus 1	AcAV1	GAAO01011981.1	462/1,052 (44%)
	Allium cepa amalgavirus 2	AcAV2	GAAN01008476.1	444/1,036 (43%)
	Blueberry latent virus	BLV	NC_014593.1	421/1,042 (40%)
	Festuca pratensis amalgavirus 2	FpAV2	GBXZ01002308.1	420/1,021 (41%)
	Festuca pratensis amalgavirus 3	FpAV3	GBXZ01009138.1	403/1,038 (39%)
	Lolium perenne amalgavirus 1	LpAV1	GAYX01076418.1	402/1,035 (39%)
	Zygosaccharomyces bailii virus Z	ZbV-Z	KU200450.1	130/521 (25%)
SpLV	Spinach latent virus	SpLV	NC_003809.1	748/804 (93%)
	Elm mottle virus	EMoV	NC_003568.1	632/798 (79%)
	Citrus variegation virus	CVV	NC_009538.1	626/800 (78%)
	Asparagus virus 2	AV2	NC_011809.1	630/798 (79%)
	Citrus leaf rugose virus	CiLRV	NC_003547.1	565/822 (69%)
	Tulare apple mosaic virus	TAMV	NC_003834.1	544/779 (70%)
	Tomato necrotic streak virus	TomNSV	KT779205.1	544/830 (66%)
	Lilac ring mottle virus	LiRMoV	EU919669.1	417/685 (61%)
	Apple mosaic virus	ApMV	NC_003465.1	230/471 (49%)

[a]Accession numbers of RNA segments encoding the RNA-dependent RNA polymerase (RdRp).

[b]Amino acid sequence identities in a format of "identical residues/aligned length (% identity)."

SpDPV1, Spinach deltapartitivirus 1; SpAV1, Spinach amalgavirus 1; SpLV, Spinach latent virus.

suggest that deltapartitiviruses have been horizontally transmitted among plants rather than having co-evolved with their hosts.

Spinach Amalgavirus 1 (SpAV1)

A sequence contig of the spinach transcriptome showed sequence similarities with Vicia cryptic virus M (VCV-M) [29], Rhododendron virus A (RHV-A) [30], and other viruses identified in the TSA database [12] (Table 2). Because these viruses are members of the genus *Amalgavirus* of the family *Amalgaviridae*, the spinach contig was named as SpAV1 and is the first *Amalgavirus* species identified in spinach. There was no sequence variation when the RNA-Seq reads of the spinach transcriptome were analyzed, indicating that SpAV1 was descended from a single virus.

The RNA genome of SpAV1 was 3,420 nt in length and contained two open reading frames (ORFs) (Table 1). ORF1 was identified to encode a 387-aa protein. The ORF1 protein showed only a limited similarity with the ORF1 proteins of other amalgaviruses, including Blueberry latent virus (BLV). However, secondary structure prediction revealed that the SpAV1 ORF1 protein was mainly composed of long α-helical regions and coils, as also observed in the other *Amalgavirus* ORF1 proteins [30].

The SpAV1 ORF2 was predicted to start at the nucleotide position 957, where the conserved motif for +1 programmed ribosomal frameshifting (PRF) was located [12]. The sequence "UUU_CGC" at the nucleotide position 953–958 matched the consensus sequence "UUU_CGN" of the +1 PRF motif found in many amalgaviruses as well as influenza A viruses (Fig. 2) [12, 31]. The +1 PRF in the ORF1+2 region produces a 1,053-aa fusion protein. The putative fusion (RdRp) protein showed around 40–50% sequence identity with the fusion proteins of amalgaviruses, including BLV and Southern tomato virus (Table 2). The SpAV1 fusion protein also showed a weak similarity to the RdRp of Zygosaccharomyces bailii virus Z (ZbV-Z), an unclassified *Amalgaviridae* species [32]. The RdRp protein sequence identity threshold for assigning amalgaviruses to different species is 65–70% [12]. Therefore, we concluded that SpAV1 is a novel species of the genus *Amalgavirus*.

A multiple sequence alignment of the fusion proteins (ORF1+2p) of SpAV1 and its related viruses revealed a highly conserved RdRp motif, which confirmed that the fusion protein was a viral RdRp [25]. A phylogenetic tree of the RdRp segments showed that SpAV1 clustered with a group of *Amalgavirus* species, among which Secale cereale amalgavirus 1 (ScAV1) was the most closely related virus

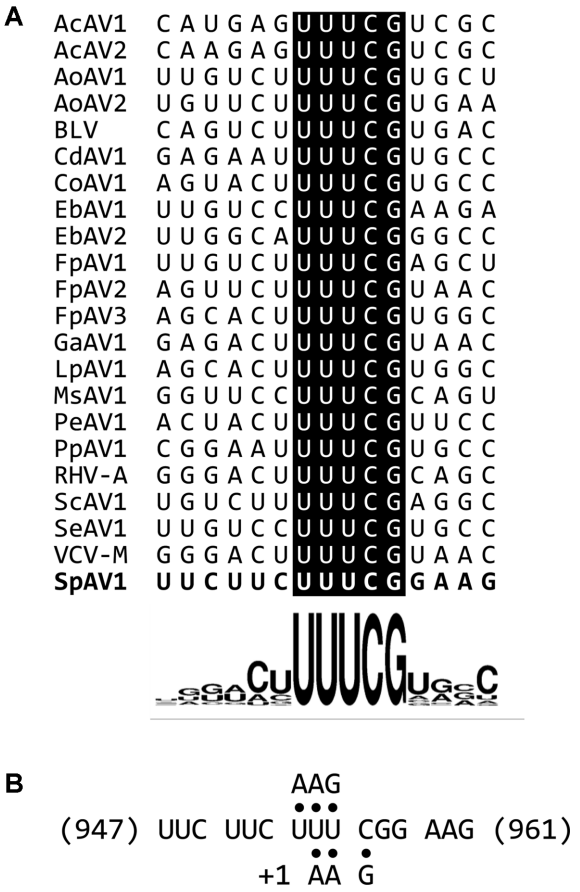

Fig. 2. Putative +1 programmed ribosomal frameshifting (PRF) motif of Spinach amalgavirus 1 (SpAV1).
(**A**) Sequences around the +1 PRF motifs of SpAV1 (bold) and other *Amalgavirus* species are shown. A sequence logo is presented at the bottom. (**B**) The +1 PRF motif of SpAV1 is shown. Both the codons UUU and UUC can be decoded by a $tRNA^{Phe}$ that has the anticodon 3′-AAG-5′. The tRNA first positioned on codon UUU may be slipped forward by one nucleotide onto the codon UUC. Codon:anticodon base pairs are marked by filled circles.

(Fig. 1B). The plant hosts of these two viruses belong to different clades of angiosperms; spinach is a eudicot and *Secale cereale* (rye) is a monocot, indicating that these viruses are dispersed by horizontal transmission among very distantly related plants.

SpLV Isolate SRP059420

The third virus identified in the spinach transcriptome consisted of three contigs, each of which showed a nucleotide sequence identity of around 99% with the corresponding genomic segment of a previously reported SpLV of the genus *Ilarvirus* of the family *Bromoviridae* [33,

34]. The newly identified SpLV was named as SpLV isolate SRP059420 or SpLV-SRP059420.

The RNA1 segment of SpLV-SRP059420 was 3,425 nt in length and encoded a 1,056-aa putative replicase that contained a methyl transferase and a helicase superfamily motif (Table 1). The RNA2 was 2,931 nt in length and had two overlapping ORFs encoding a 797-aa putative polymerase that had an RdRp motif and a 195-aa 2b protein of unknown function. The RNA3 was 2,296 nt in length and produced two proteins, a 282-aa movement protein and a 218-aa CP. The SpLV-SRP059420 ORFs showed around 99% nucleotide sequence identities to respective ORFs of the previously reported SpLV [33, 34]. There was no sequence variation in the three genomic segments when the RNA-Seq reads were analyzed, suggesting that the assembled genomic segments were derived from a single clone.

The 5′-UTRs of the SpLV-SRP059420 segments RNA1 and RNA2 exhibited a strong sequence similarity to each other, whereas no similarity was found with the RNA3 segment. The 3′-UTRs of all the three segments, however, showed a high level of sequence similarity among them. These features are commonly observed in ilarviruses [33, 35].

The SpLV-SRP059420 polymerase, which contained an RdRp motif, showed 61–93% aa sequence identities with the polymerases of the previously reported SpLV, Elm mottle virus (EMoV), Citrus variegation virus (CVV), Asparagus virus-2 (AV2), Citrus leaf rugose virus (CiLRV), Tulare apple mosaic virus (TAMV), Tomato necrotic steak virus (TomNSV), and Lilac ring mottle virus (LiRMoV) (Table 2). These viruses are members of the genus *Ilarvirus* subgroup 2. Apple mosaic virus (ApMV), a member of the *Ilarvirus* subgroup 3, showed a weaker similarity. A phylogenetic tree inferred from the RdRp sequences confirmed that SpLV clustered in *Ilarvirus* subgroup 2, with LiRMoV being the most distant member (Fig. 1C).

In conclusion, three viruses (SpDPV1, SpAV1, and SpLV-SRP059420) with complete protein-coding regions were identified from a spinach transcriptome dataset. Sequence similarity and phylogenetic analyses showed that SpDPV1 and SpAV1 were novel virus species that belonged to the genera *Deltapartitivirus* and *Amalgavirus*, respectively. This is the first report of these virus species in spinach. The approach developed in this study can be applied to other transcriptome data to identify persistent RNA viruses. In fact, preliminary investigation of transcriptome data obtained from various plants, including ginseng and eelgrass, have yielded several potentially novel RNA virus genome sequences.

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