Characterization of a new apple luteovirus identified by high-throughput sequencing

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Abstract

Background: ‘Rapid Apple Decline’ (RAD) is a newly emerging problem of young, dwarf apple trees in the Northeastern USA. The affected trees show trunk necrosis, cracking and canker before collapse in summer. In this study, we discovered and characterized a new luteovirus from apple trees in RAD-affected orchards using high-throughput sequencing (HTS) technology and subsequent Sanger sequencing.

Methods: Illumina NextSeq sequencing was applied to total RNAs prepared from three diseased apple trees. Sequence reads were de novo assembled, and contigs were annotated by BLASTx. RT-PCR and 5’/3’ RACE sequencing were used to obtain the complete genome of a new virus. RT-PCR was used to detect the virus.

Results: Three common apple viruses and a new luteovirus were identified from the diseased trees by HTS and RT-PCR. Sequence analyses of the complete genome of the new virus show that it is a new species of the genus Luteovirus in the family Luteoviridae. The virus is graft transmissible and detected by RT-PCR in apple trees in a couple of orchards.

Conclusions: A new luteovirus and/or three known viruses were found to be associated with RAD. Molecular characterization of the new luteovirus provides important information for further investigation of its distribution and etiological role.

Keywords: Rapid apple decline, USA, Luteovirus, Genomic sequence

Background

Apple (Malus domestica L.) is the most widely cultivated fruit crop worldwide [1]. The U.S. is the world’s second-largest producer of apple with a wholesale value of $4 billion (https://www.usapple.org/all-about-apples/apple-industry-statistics/). Apple is propagated by grafting, budding and layering. The careless selection of infected materials for the propagation allows the accumulation of virus/viruses in apple trees and dissemination of viruses between trees, orchards and regions. At least ten viruses and four viroids have been reported to infect apple trees, causing many types of diseases that reduce fruit quality and yield [2]. Among the most commonly reported viruses are apple stem pitting virus (ASPV), apple stem grooving virus (ASGV) and apple chlorotic leaf spot virus (ACLSV), all species of the family Betaflexiviridae. These viruses are ubiquitous and frequently occur as mixed infections. They are commonly called latent viruses because they usually do not induce obvious symptoms in most cultivars used in production, although yield reductions have been reported [3].

For the last several years, an unusual problem of young, apple trees growing of dwarfing rootstock in the northeastern U.S. has been observed (https://extension.psu.edu/apple-disease-rapid-apple-decline-rad-or-sudden-apple-decline-sad). The problem has been named ‘Rapid Apple Decline’ (RAD) or ‘Sudden Apple Decline’ due to the rapid or sudden death of apple trees after the first appearance of symptoms (Fig. 1). Several scion cultivars start to decline after grafting onto the M9 rootstock. The affected trees usually exhibit cankers and cracks on the rootstock and/or scion trunks. Necrosis begins at the graft union and proceeds up the trunks. The leaves of some affected trees begin...
to look yellow and then redden, and within two weeks the
trees collapse from late July through September.

The involvement of fire blight and other pathogens
[phytophthora and tomato ringspot virus (ToRSV)],
herbicide damage, winter and drought injuries in RAD
has been largely ruled out through rigorous observations
and/or testing. This prompted us to use high-
throughput sequencing (HTS) to further investigate pos-
sible causal agent(s) of RAD.

HTS combined with bioinformatic analyses is becom-
ing a routine technology in the field of plant virology for
the discovery of many new and emerging viruses, detec-
tion of known viruses and investigation of viral genetic
diversity and evolution [4]. In this study, Illumina RNA
sequencing technology was used to identify the patho-
gens potentially associated with RAD, including a new
luteovirus tentatively named apple luteovirus 1.

Methods
Sample collections and preparations
In June 2016, samples of branches were collected from
six RAD symptomatic apple trees (Table 1; PA2, PA4,
PA5, PA7, PA8 and PA9) in a 5-year-old orchard at the
Pennsylvania State University Fruit Research and Exten-
sion Center (PSU-FREC). The apple cultivar was Crim-
sen Crip grafted on M9 rootstock. Two samples, one
from scion and another from rootstock suckers, were
collected from each tree. For HTS analysis, total nucleic
acids were extracted from leaf, petiole and bark tissues
by a CTAB method [5] and used for total RNA isolation
by RNeasy<sup>®</sup> Plant Mini Kit (Qiagen, Germantown, MD).
TNA by the CTAB method were used for the detection
of all four viruses.

High-throughput sequencing and analyses
Total RNAs of three pooled samples, CPAR (all 6 root-
stock samples), CPAS1 (scion samples of PA2, PA4 and
PA5) and CPAS2 (scion samples of PA7–9), were proc-
essed at SeqMatic (Fremont, CA). Plant ribosomal RNAs
(rRNA) were removed from total RNAs using Illumina
Ribo-Zero rRNA Removal Kit for cDNA library con-
struction. The samples were sequenced on the Illumina
NextSeq sequencing platform with 15-sample bar-coded
multiplexing.

Analyses of total sequence reads were performed using
the CLC Genomics Workbench 9.5.2 platform (https://
www.qiagenbioinformatics.com/). The raw reads were
filtered to remove the failed reads, and qualified reads
were assembled de novo into contigs with a cut-off of
150-nt. Contigs were annotated by BLASTx comparisons
to Viruses_NR and Viroids databases downloaded from
NCBI GenBank databases.

Validation of the viruses and genome determination of a
new luteovirus
To verify the presence of a new luteovirus in the six
samples used in HTS, RT-PCR using primers AluDetF6/
R6 (Additional file 1) were used. The three latent viruses
were also detected by RT-PCR using virus-specific
primers (Additional file 1) designed based on alignments
of their genomic sequences available in GenBank. The
RT-PCR was performed using the SuperScript™ III One-
Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) in
a 20-μl reaction containing 1 μl of TNAs, 1.0 μl of each
primer (5 μM), 10 μl of 2× Reaction Mix, 0.4 μl of En-
zeyme Mix and 6.6 μl of water. The thermal cycling con-
ditions for RT-PCR were 1 cycle of 50 °C for 30 min and
94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55–60 °C
(varied according to the primer pairs) for 1 min and 68 °C for 40 s and one final extension at 68 °C for 5 min.

To obtain the complete genomic sequence of the new luteovirus, primers were designed based on the contig sequences with similarity to several luteoviruses (Additional file 1). The TNAs of the PA8 sample (Table 1) was used as template in the RT-PCR. The 5′-end sequence was obtained by a 5′RACE System Kit (Invitrogen). The 3′-end sequence was determined by a First Choice RLM-RACE Kit (Invitrogen) after polyadenylation of the RNAs using poly(A) polymerase (New England BioLabs, Ipswich, MA). All amplicons were cloned into pGEM-T Easy Vector (Promega, Madison, WV), and plasmid DNAs isolated from overnight cultures were sequenced (MCLAB, San Francisco, CA).

**Genomic sequence analysis of the luteovirus**

The sequences were assembled and analyzed by the CLC Genomics Workbench. Open reading frames (ORFs) were predicted using the Open Reading Frame Finder at [https://www.ncbi.nlm.nih.gov/orffinder/](https://www.ncbi.nlm.nih.gov/orffinder/). Multiple alignments of genomic sequences and deduced amino acid sequences of individual viral genes were performed by the neighbor-joining algorithm as implemented in ClustalW, and the resulting alignments were analyzed using MEGA7 [6]. Recombination analysis was carried out using RDP 4.83 package [7].

**Graft transmission of the luteovirus**

To verify the luteovirus sequences are associated with graft-transmissible agents, dormant buds of each of four selected trees from the PSU block (Table 1; PA13, PA14, PA18 and PA21) were grafted onto seven apple seedling trees. All seedlings tested negative for the luteovirus and three latent viruses. PA21 is infected with the luteovirus alone, but PA13, PA14 and PA18 are mix infected with at least one latent virus. Leaves were collected from new shoots of each of the inoculated seedlings at one, five and seven months after grafting. TNA were extracted from leaf and petiole tissue and used as template in by RT-PCR as described above. An uninoculated apple seedling was used as negative control.

**Additional detection of the luteovirus**

Eighty samples were collected from both RAD symptomatic and symptomless apple trees from PSU-FREC

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**Table 1** Cultivars, symptoms and viruses of apple trees in a research block

| Sample | Cultivar | Symptoms | Virus | ALV | ACLSV | ASGV | ASPV |
|--------|----------|----------|-------|-----|-------|------|-------|
| PA2    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | +   | +     | +    |
| PA4    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | –   | +     | +    |
| PA5    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | +   | –     | +    |
| PA7    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | –   | +     | –    |
| PA8    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | –   | –     | +    |
| PA9    | Crimson Crisp | Trunk cracking, leaf yellowing | +     | –   | –     | –    |
| PA11   | Crimson Crisp | NS       | +     | +   | +     | +    |
| PA12   | Crimson Crisp | NS       | +     | +   | +     | +    |
| PA13   | Crimson Crisp | Bark cracking | +     | +   | +     | +    |
| PA14   | Crimson Crisp | NS       | +     | +   | +     | +    |
| PA15   | Crimson Crisp | Upper branch browning | +     | +   | –     | +    |
| PA16   | Crimson Crisp | NS       | +     | +   | +     | +    |
| PA17   | Crimson Crisp | Leaf distortion, tree dying | +     | +   | +     | +    |
| PA18   | Fuji      | Trunk cracking, leaf curl | +     | –   | +     | –    |
| PA19   | Fuji      | NS       | –     | –   | –     | –    |
| PA20   | Gala      | Trunk cracking, small leaves | +     | +   | +     | –    |
| PA21   | Fuji      | NS       | +     | –   | –     | –    |
| PA22   | Gala      | NS       | +     | –   | –     | +    |
| PA23   | Golden Delicious | Trunk cracking, leaf curl | +     | –   | +     | –    |
| PA24   | Golden Delicious | NS       | +     | –   | –     | –    |

*Samples PA2–9 were collected in June 2016

All cultivars were grafted on M9 rootstock

NS mean that there were no obvious symptoms

ALV 1 Apple luteovirus 1, ACLSV Apple chlorotic leaf spot virus, ASGV Apple stem grooving virus, ASPV Apple stem pitting virus
and USDA-ARS Appalachian Fruit Research Station (AFRS) in West Virginia (Table 2). The FREC trees in Pennsylvania were grafted on M9 rootstock, whereas the AFRS trees were grafted on EMLA 7 rootstock. A symptomatic tree (PA14) infected with the luteovirus and three latent viruses and an apple seedling were used as controls. RNAs prepared from leaves, petioles and bark were used as templates in RT-PCR, as described above. Viral amplicons of selective samples of each location were cloned and sequenced by Sanger sequencing.

**Results**

**Virus identification by high-throughput sequencing**

Total reads of 27,727,559 (CPAR), 28,817,295 (CPAS1) and 30,614,167 (CPAS2) were obtained after removing the failed reads. Assembly de novo of the reads generated a total of 59,415 (CPAR), 60,992 (CPAS1) and 65,115 (CPAS2) contigs larger than 150 nt. Blastx searches against the Viruses_NR database revealed contigs with amino acid (aa) sequence identities of 29–76% to peach associated luteovirus (PaLV), cherry associated luteovirus (ChaLV) and rose spring dwarf associated virus (RSDaV) of the genus *Luteovirus* in the family *Luteoviridae* from CPAR (2 contigs), CPAS1 (6 contigs) and CPAS2 (2 contigs), respectively. Approximately 335,446 (86×), 48,908 (12×) and 778,086 (199×) reads were mapped to contig CPAS1–8, the longest luteovirus contig, supporting the presence of the virus in the three samples. Multiple contigs with identities of 83–99% to ACLSV, ASGV and ASPV were also identified from all three samples, respectively, but the data are not presented here. No viroid was detected from any of the HTS samples.

**RT-PCR detection the viruses in orchard samples**

The luteovirus was detected in all six HTS samples (6/6, 100%), whereas ACLSV (2/6, 33%), ASGV (4/6, 67%) and ASPV (4/6, 67%) were only found in some of these trees (Additional file 2). PA9 was infected with the luteovirus alone, whereas five other trees were infected with at least one latent virus. Fourteen more samples consisting of four different cultivars were collected from the same PSU-FREC orchard and tested by RT-PCR. The luteovirus was detected in 13 of them (93%) (Table 1). The latent viruses were again detected from fewer samples (ACLSV 64%, ASGV 64% and ASPV 57%).

To expand the testing for the luteovirus, a total of 80 additional samples were collected from the PSU-FREC and AFRS orchards (Table 2). Some trees in the PSU-FREC orchard showed the disease symptoms, whereas those in the AFRS orchard did not have obvious symptoms. Results of RT-PCR showed that majority of cv. Fuji trees (11/12) and a small number of cv. Gala (2/12) and Gold Delicious (2/12) trees in the PSU-FREC orchard were infected with ALV-1, but the infection rates were much lower for the cultivars in the AFRS orchard.

**Graft transmission**

The graft inoculated apple seedlings did not display obvious symptoms nine months after inoculation. Infections of the luteovirus and latent viruses were confirmed by RT-PCR using specific primers, respectively (Additional file 1). A weak amplification was obtained from the PA21 seedlings one month after inoculation (data not shown), and all four samples tested positive five months after inoculation (Additional file 3).

**Complete genome of the luteovirus**

Complete genome of the new virus, with the proposed name apple luteovirus 1 (ALV-1), is 6001 nucleotides (nt) in size (GenBank no. MF120198), encoding ten open reading frames (ORFs). The genome starts with a conserved element GTGAUU^6 (underlined nt is different from other species of *Luteovirus*) and contains all cis-acting elements of the luteoviruses [8, 9]. The conserved GGAGGAGAGGAGGCU^1380 and CC CGGC UUGAAGCCCUUU^1410 known to be responsible for the −1 ribosomal frameshift are located at the junction of ORF1 and ORF2. A tract of ten tandem CCXXX (X is any base) repeats that is required for the ORF3 stop

| Location | Collection date | Cultivar | Number of samples | Infection rate |
|----------|-----------------|----------|------------------|----------------|
| PSU-FREC | 09/16/2014      | Fuji     | 12               | 11 (91.6%)     |
| Pennsylvania |               | Gala     | 12               | 2 (16.7%)     |
| AFRS     | 01/31/2017      | Crimson Crisp | 13              | 4 (30.7%)     |
| West Virginia |             | Fuji     | 11               | 1 (9.1%)      |
|           |                 | Hampshire| 10               | 0             |
|           |                 | Snapp Stayman | 10              | 1 (10.0%)     |
| Total    |                 |          | 80               | 21 (26.3%)    |

^PSU-FREC Pennsylvania State University-Fruit Research and Extension Center, AFRS Appalachian Fruit Research Station

^Cultivars in the PSU-FREC orchard were grafted on M9 rootstock, and cultivars in the AFRS orchard were grafted on EMLA 7 rootstock
codon readthrough starts at nt 3695. The 3′ terminal region contains all conserved elements [8] but the longest motif at the first stem-and-loop of the barley yellow dwarf virus-like element (BTE) is different as GUACGUCCUGGUAAGACAGG (bolded and italicized nt represents inserted nt). These two insertions, CGCG and 516G, are unique to ALV-1.

The arrangement and structure of six of these ORFs (ORF1, ORF2, ORF3, ORF3a, ORF4 and ORF5) resembles that of other luteoviruses (Fig. 2a) [8, 10–14]. The ORF1 and ORF2 encode putative P1 and P1-P2 fusion proteins (by the −1 frameshift translation), respectively, and together they form a putative replicase complex. The ORF3 encodes a putative coat protein (CP), and translation via read-through of its stop codon produces a putative P3-P5 fusion protein that might be involved in insect transmission. The ORF4 encodes a putative movement protein (MP). Like most luteoviruses, ALV-1 also encodes a small ORF3a (nt 2956–3093) that is essential for long distance movement [15]. ORF6 and ORF7 are only present in some luteoviruses [11, 13, 14], and ORF1a and ORF5a are unique to this virus. ORF1a embedded within ORF1 is in a similar position to ORF0 of the genus Enamovirus [16] but its gene product (264 aa residues) does not share any sequence similarity to the enomovirus ORF0. ORF5a within ORF5 encodes a putative protein of 96 aa residues. The putative proteins encoded by ORF1a and ORF5a have no sequence homology with any known proteins.

Sequence comparisons and phylogenetic analysis of the luteovirus

Comparisons of genomic and individual protein sequences among species in the family Luteoviridae confirmed that ALV-1 is most closely related to PaLV (Fig. 2b). The genome sequence identities between the two viruses is 53% at the nucleotide sequence level, which falls within range of 48–69% among the luteoviruses [17]. Sequence comparisons showed that the P1-P2 replicase was the most conserved (52–63%), whereas the P4 (MP) was the least conserved (27–39%) between ALV-1 and known luteoviruses at aa sequence level. Except for the P3a, ALV-1 had the highest aa sequence identity with PaLV or ChALV at the individual proteins. The P3a of ALV-1 was most closely related to soybean mosaic virus (SMV). According to the species demarcation criteria for the family Luteoviridae (≥10% difference in aa sequence

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**Fig. 2** Genomic organization of apple luteovirus 1 (a) and its sequence identity percentages with other viruses of the genus Luteovirus and representative viruses of other genera in the family Luteoviridae (b). BTE, barley yellow dwarf virus-like translational element; n/a, data not available. The numbers below each open reading frame (ORF) box indicate the positions of the start and stop codons of each ORF, respectively.

| Virus                                      | Genome (6001 nt) | P1 (424 aa) | P1-P2 (953 aa) | P3a (45 aa) | P3 (200 aa) | P4 (174 aa) | P3-P5 (780 aa) |
|--------------------------------------------|------------------|-------------|----------------|-------------|-------------|-------------|---------------|
| Peach associated luteovirus                | 52.8             | 46.4        | 63.1           | 26.7        | 45.7        | 37.4        | 39.7          |
| Cherry associated luteovirus               | 52.5             | 44.9        | 62.7           | 26.1        | 45.5        | 38.7        | 40.7          |
| Rose spring dwarf-associated virus         | 52.5             | 38.7        | 53.9           | 60.0        | 40.2        | 26.6        | 36.2          |
| Nectarine stem pitting-associated virus    | 49.3             | 30.7        | 54.2           | n/a         | 38.9        | n/a         | 32.3          |
| Barley yellow dwarf virus Ker-II           | 49.4             | 33.8        | 52.4           | 42.2        | 40.8        | 30.7        | 35.1          |
| Barley yellow dwarf virus Ker-III          | 51.6             | 30.7        | 52.9           | n/a*        | 42.5        | 34.0        | 35.1          |
| Barley yellow dwarf virus-GAV              | 50.5             | 33.7        | 52.2           | 44.4        | 41.7        | 34.7        | 35.2          |
| Barley yellow dwarf virus-MAV              | 50.1             | 33.4        | 52.6           | 46.7        | 41.7        | 34.0        | 55.3          |
| Barley yellow dwarf virus-PAS              | 50.5             | 34.7        | 52.7           | 44.4        | 44.0        | 37.3        | 35.1          |
| Barley yellow dwarf virus-PAV              | 50.6             | 33.7        | 52.2           | 44.4        | 43.5        | 36.0        | 34.9          |
| Bean leaf roll virus                       | 49.1             | 39.0        | 56.9           | 43.2        | 36.3        | 31.8        | 30.8          |
| Soybean dwarf virus                        | 49.6             | 38.7        | 55.7           | 51.1        | 36.5        | 34.9        | 31.3          |
| Citrus vein enation virus                  | 39.9             | 11.1        | 17.4           | n/a*        | 18.7        | n/a         | 33.1          |
| Sugarcane yellow leaf virus                | 38.8             | 15.6        | 17.7           | 30.1        | 19.3        | 34.9        | 30.3          |

1. nt = nucleotides; 2. aa = amino acids
of any gene) [17], ALV-1 should be a new species of the genus Luteovirus.

Phylogenetic analyses conducted using the genomic sequences of ALV-1 and other luteoviruses placed the virus with ChALV, PaLV, RSDaV and nectarine stem pitting-associated virus (NSPaV) in a cluster distinct from that of BDYVs and two legume luteoviruses (Fig. 3). Topologies of phylogenetic trees changed slightly when the aa sequences of the P1-P2 and CP aa sequences were analyzed but close relationship of the four viruses were retained (data not shown). Analysis of the genomic sequences of 45 species of the family Luteoviridae by RDP4 did not detect any recombination breakpoints.

**Discussion**

A novel luteovirus provisionally named apple luteovirus 1 and three common viruses (ACLSV, ASGV and ASPV) were identified by HTS of total RNA extracted from RAD-affected apple trees. BLAST search in pathogen databases identified several contigs showing similarities with members of the genus Luteovirus in the family Luteoviridae, particularly with four known luteoviruses (ChALV, NSPaV, PaLV and RSDaV) that infect woody plants [11–14]. The 6001-nt genomic sequence of ALV-1 is the largest genome of the known luteoviruses due to insertions in ORF1, ORF3 and ORF5. The arrangement and structure of the ALV-1 genome resembles those of other luteoviruses, containing six hallmark ORFs of the genus (Fig. 2a), encoding proteins involved in replication (P1 and P1-P2 fusion protein), virion assembly (CP), movement (P3a and P4) and aphid transmission (P3-P5 fusion protein) [9–11]. The ALV-1 genome also has several unique features. The 17-nt BTE motif conserved in the 3’ terminus of all known luteoviruses changes to 5’-GUACGUCCUGGUAGAACAGG-3’ in ALV-1 due to two insertions (bolded and italicized bases), making it a unique motif among the luteoviruses. The ALV-1 genome also contains two additional ORFs (1a and 5a) that are not present in any known luteoviruses. Pairwise comparisons showed that ALV-1 has the highest genomic sequence identity (52.8%) with PaLV [14], indicating ALV-1 is a distinct species of the genus Luteovirus. Phylogenetic analysis also placed ALV-1 with the woody plant-infecting luteoviruses, suggesting that these viruses share a common ancestor.
Recombination plays an important role in the evolution of plant RNA viruses. Both bean leafroll virus and SMV are recombinants that occurred before the species separation [17]. It is a pervasive phenomenon among BYDV species and isolates [18]. However, recombination analysis did not reveal any recombination events in the ALV evolution.

Graft inoculation of apple seedlings demonstrated that ALV-1 is graft transmissible, and the virus could be spread by vegetative propagation of scions or rootstock or both. The source of this luteovirus is unknown, and subsequent study of rootstock and mother trees of different apple cultivars is necessary to determine the original infection.

Symptoms of RAD are very similar to apple union necrosis and decline (AUND) described in New York in early 1980s [19]. The eight apple cultivars propagated on MM106 rootstock showed graft union necrosis and tree decline. Similar diseases such as citrus tristeza quick decline disease (T-QD) [20] and citrus sudden death (CSD) have described in citrus trees [21]. T-QD destroyed millions of sweet citrus trees (Citrus sinensis L. Osb.) propagated on sour citrus rootstock (C. aurantium L.) worldwide many years ago [19], and CSD has started to kill hundreds of thousands of sweet oranges propagated on Pangpur lime rootstock (C. limonia L. Osb.) in Brazil since 1999 [20]. T-QD is caused by citrus tristeza virus (CTV), and CSD has been associated with citrus sudden-death associated virus (CSDaV), a species of the genus *Marafivirus* in the family *Tymoviridae*. RAD is only observed on apple trees grafted on certain dwarf rootstock cultivars such as M9. Both T-QD and CSD also occurred only on sweet orange grafted on certain rootstock [20, 21]. T-QD was controlled by replacement of the sour orange rootstock with CTV-tolerant rootstock [20]. Further investigation of ALV-1 infection in different rootstocks and scion-rootstock combinations is necessary to understand the role of susceptible rootstocks in RAD.

Nothing is known about the vector transmission of ALV-1. Salem et al. [22] reported that RSDaV is transmitted by rose-grass aphid (Metopolophium dirhodum Walker) and yellow rose aphid (Rhodobium porosum Sanderson). The aphid transmission of ChALV, NSPaV and PaLV has not been studied yet. Although aphids were observed in some RAD-affected orchards, their role in spreading ALV-1 in orchards needs to be defined.

Koch's postulates have not yet been fulfilled so we cannot yet conclude that ALV-1 is a causal agent of RAD. The ALV-1 infection in the PSU-FREC orchard affected with RAD is very common, with 93% of the trees tested positive for ALV-1 and 57–64% tested positive for the three apple latent viruses (Table 1). Mix infection of the viruses in the RAD-affected orchard makes it difficult to associate ALV-1 with RAD. We are conducting bud grafting inoculation of ALV-1 on few apple cultivars grafted on M9 and other rootstocks to determine the role of ALV-1 in RAD.

The identification of a novel luteovirus in apple broadens the host range of the luteoviruses. Detecting ALV-1 from the RAD-affected trees justifies investigations of the role of this virus in the etiology of this newly emerging problem. The genomic sequence of ALV-1 obtained in this study enables the development of a specific RT-PCR for the rapid detection of the virus. This is
important to study the distribution, transmission and pathogenicity of ALV-1.

Conclusion
This paper reports the identification of a new luteovirus and/or three known viruses associated with RAD in Northeast USA. The genome of apple luteovirus 1 was obtained, and the virus was proven to be transmitted by grafting. These data provide important information for further investigation of the role of ALV-1 in RAD.

Additional files

Additional file 1: Primers used in this study. (DOCX 13 kb)

Additional file 2: Virus detection by RT-PCR in RAD-affected apple trees used for high-throughput sequencing. The four viruses are apple luteovirus 1 (ALV-1), apple chlorotic leaf spot virus (ACLSs), apple stem greening virus (ASGV) and apple stem pitting virus (ASPV), respectively. Lanes M 1 kb plus DNA ladder, and W water. Arrow indicate the DNA fragment with labeled size. (PPTX 2.26 kb)

Additional file 3: Verification of graft transmission of apple luteovirus 1 to apple seedlings by RT-PCR using primers AluDetF6/R6 (Additional file 1). Lanes M 1 kb plus DNA ladder; 1–3 from PA13; 4–6 from PA14; 7–9 from PA18; 10–12 from PA21; 13 PA14; 14 water. Arrow indicate the DNA fragment with labeled size. (PPTX 157 kb)

Abbreviations
ACLSs: Apple chlorotic leaf spot virus; ALV-1: Apple luteovirus 1; ASGV: Apple stem greening virus; ASPV: Apple stem pitting virus; ChaLV: Cherry associated luteovirus; HTS: High-throughput sequencing; NSPv: Nectarine stem pitting-associated virus; nt: Nucleotide; ORF: Open reading frame; PaLV: Peach associated luteovirus; RAD: Rapid apple decline; RSDaV: Rose spring dwarf-associated virus

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
Conceived and designed the experiments: RL, HWL, LPW. Performed the experiments: HLW, LPW, EN, KP. Analyzed the data: RL, HLW, LPW. Contributed reagents/materials/analysis tools: ZL, DM, MJ. Wrote the paper: RL, HLW. Supervised the study: RL. Edited the final manuscript: EN, KP, ZL. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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