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Development and characterization of the first infectious clone of alfalfa latent virus, a strain of *Pea streak virus*

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Abstract Alfalfa *(Medicago sativa*) is a natural host plant for many plant pathogens including fungi, bacteria, nematodes and viruses. Alfalfa latent virus (ALV) is strain of *Pea streak virus*, a member of the carlavirus group that occurs symptomlessly in alfalfa. The first complete genomic sequence of the ALV that was recently obtained in our laboratory showed that the virus differs substantially from other members of the genus *Carlavirus*. Here we report generation of infectious RNA transcripts from the constructed full-length viral cDNA clone as a proof that ALV nucleotide sequence is correct and as an initial step toward development of the ALV-based vector for gene silencing and expression of foreign proteins in alfalfa. This is the first report describing the development of a complete cDNA clone of the ALV strain of *Pea streak virus* and its infectivity in the diagnostic pea (*Pisum sativum*) and natural alfalfa hosts.

Keywords Alfalfa latent virus · Full-length infectious cDNA clone

Alfalfa (*Medicago sativa*) is a key forage crop for dairy producers in the US and in many countries of the world. It is an important component of sustainable agricultural systems because of its high yield, value for soil conservation, N_2 fixation, crop rotation and wildlife habitat (Putnam et al. 2001). Alfalfa genomics is in the early stages of development primarily due to the complex genetic structure of this autotetraploid. Alfalfa is a natural host plant for many plant pathogens including fungi, bacteria, nematodes and viruses. One of the low-impact viral pathogens infecting alfalfa is alfalfa latent virus (ALV). ALV was first described as a separate species and a new member of the carlavirus group that occurs symptomlessly in alfalfa (Veerisetty and Brakke 1977). It was later recognized to be a mild strain of *Pea streak mosaic virus*, genus *Carlavirus*, family *Betaflexiviridae*, due to their serological cross-reactivity and particle length (Hampton 1981). In the United States, the virus is prevalent in Nebraska and Wisconsin (Veerisetty 1979). The first complete genomic sequence of the ALV strain of pea streak mosaic virus (PeSV) recently determined in our laboratory, showed that the virus differs substantially from other members of the genus *Carlavirus* (Nemchinov et al. 2015). Here we report generation of infectious RNA transcripts from the full-length viral cDNA as a proof that ALV nucleotide sequence is accurate and as the first step toward development of the ALV-based vector for gene silencing and expression of foreign proteins in alfalfa. Up to now, virus-induced gene silencing (VIGS), one of the most widely used tools in plant functional genomics, has not been applied toward alfalfa research due to lack of appropriate virus vector. Availability of specific VIGS vector would greatly facilitate studies of the key genes involved in various aspects of alfalfa development and adaptation to the environment.

Electronic supplementary material The online version of this article (doi:10.1007/s10658-017-1237-2) contains supplementary material, which is available to authorized users.

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ALV-infected tissues used in this study were obtained from the ATCC under APHIS permit # P526P-14-00733 (PV-264 isolate from Lancaster County, NE, USA). Total RNA was extracted with the TRIzol protocol (Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer's directions. The full-length ALV cDNA including 5′ and 3′ termini has been prepared as two fragments, KpnI-AgeI and AgeI-NotI, which were then assembled within the pUC57(Kan) plasmid by Medigen, Inc. (Frederick, MD) for a fee. All clones were sequence-verified. The constructs included 30 bp-long polyA tail at the 3′ terminus preceding NotI restriction site.

The full-length ALV cDNA in the pUC57 plasmid (pALV) contains the T7 RNA Polymerase promoter incorporated upstream of the 5′ end of the viral sequence (Fig. 1 and Supplementary Material Fig. S1). Plasmids were linearized with the NotI restriction enzyme and capped transcripts were generated in vitro from cDNA clones using Ambion's T7 mMessage Machine or Megascript T7 Transcription kits as advised by manufacturer (Thermo Fisher Scientific, Inc., Waltham, MA). Transcripts were rub-inoculated onto fully expanded leaves of two-week old *Pisum sativum* cv. Lincoln seedlings dusted with carborundum powder. Inoculated plants were kept in a containment greenhouse facility of the Molecular Plant Pathology Laboratory under 16-h day-light period.

Within three weeks after inoculation, pea plants developed chlorosis which became obvious one month after inoculation. Chlorotic leaves often had necrotic symptoms along their margins (Fig. 2). Western blot (WB) assay was performed with symptomatic leaves of *P. sativum* as described in Nemchinov and Natilla (2007). Membranes were probed with polyclonal antibodies to PeSV (AC Diagnostics, Inc., Fayetteville, AR). The serological test showed that pea plants are infected with the virus (Fig. 3a). Plant tissues of *P. sativum* were also examined by transmission electron microscopy (TEM). For TEM, leaves were homogenized in sterile water following by centrifugation of the extracts in a bench-top Eppendorf centrifuge for three minutes at 16.1 rcf (13.2 krpm). Virus captured on the TEM grids was stained with 1% Phosphotungstate (PTA) solution. The grids were examined in the Hitachi H-7700 Electron Microscope at the Electron and Confocal Microscope Unit, Beltsville Agricultural Research Center. Flexuous virus particles with the modal length characteristic for ALV (636–640 nm) were observed in the extracts from the infected pea plants (as shown in Fig. 3b for the purified preparation).

Following detection of the pathogen by WB and TEM, the virus was purified from the transcript-inoculated plants of *P. sativum* in order to obtain contaminant-free virus inoculum suitable for infection of alfalfa, since inoculation of alfalfa with crude extracts was not successful. TEM-verified infected plants of *P. sativum* cv. Lincoln were removed from soil and stored at −20 °C until virus purification. The virus was purified from ~85 g of whole pea plants (excluding roots) exactly as described in Veerisetty and Brakke (1978). Virus concentration estimated from the absorbance at 260 nm using NanoDrop ND 2000 spectrophotometer (Thermo Scientific) was ~3.97 mg/ml, assuming that extinction coefficient (E) for 0.1% (mg/ml) solution at 260 nm = 3.0 (Veerisetty and Brakke 1978). Purified viral preparations examined by TEM contained flexuous filaments without any plant contaminants (Fig. 3b) and reacted positively with antiserum to PeSV in WB assays (not shown). To further confirm the identity of the virus, an RT-PCR was performed with the purified preparations using the following primers: 5′ CGATTGTGTCTCTGGTCATCTC 3′ (forward primer, position 6400–6421) and 5′ ATAAAGATGGCAGA

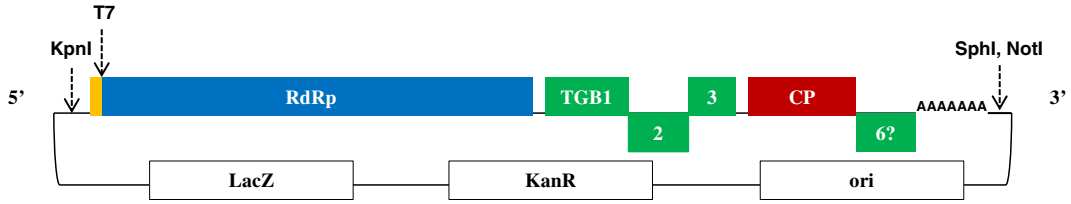

Fig. 1 Full-length ALV cDNA clone. T7: T7 RNA Polymerase promoter; RdRP: RNA-dependent RNA polymerase; TGB1, 2 and 3: triple gene block protein 1, 2 and 3, CP: capsid protein. 6?: putative nucleotide-binding protein (ORF 6). LacZ, KanR and ori from pUC57 plasmid backbone

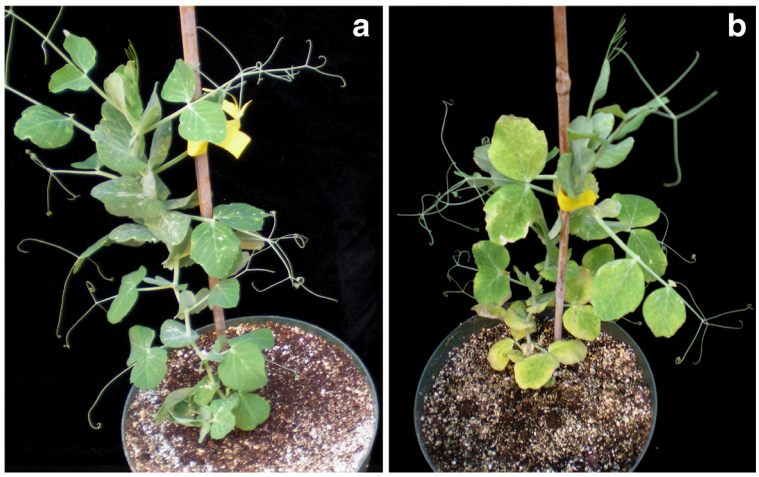

Fig. 2 Symptoms of alfalfa latent virus on transcript-inoculated plants of *Pisum sativum* cv. Lincoln. **a** Non-inoculated plant. **b** Plant inoculated with transcripts derived from the full-length cDNA clone of ALV

GCAACAGA 3′ (reverse primer, position 7062–7083, GenBank accession number KP784454). The procedure, named simple-direct-tube RT-PCR (SDT-RT-PCR) was carried out essentially as described by Suehiro et al. (2005). It led to amplification of PCR product of the expected size (684 bp) from all purified viral samples (Supplementary Material Fig. S2). Sequencing demonstrated that the amplified DNA segments corresponded to the targeted area of the ALV genome.

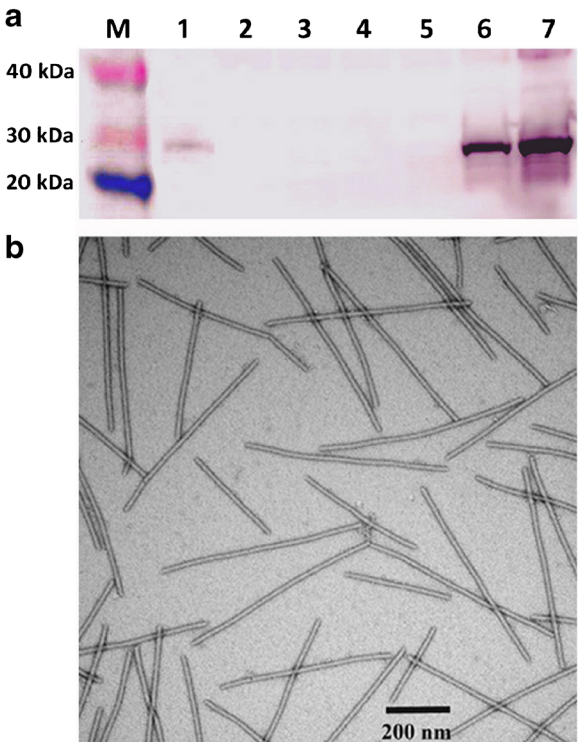

Fig. 3 **a** Western blot assay performed with symptomatic leaves of *P. sativum* inoculated with RNA transcripts. Membrane was probed with polyclonal antibodies to PeSV. M, Color Burst electrophoresis marker (Sigma-Aldrich). Lane 1, ALV positive control (1:10 dilution of the extracts from ALV-infected tissues obtained from ATCC); Lanes 2–5, uninfected *P. sativum* plants. Lanes 6 and 7: ALV-infected *P. sativum* plants. **b** ALV preparation purified from the infected plants of *P. sativum* cv. Lincoln. Scale bar represents 200 nm

Two-week old alfalfa seedlings of Regen SY germplasm (Bingham 1991), acquired from the collections of National Plant Germplasm System were rub-inoculated with purified viral preparations obtained from infected pea plants. Two weeks after inoculation, the virus was detected in non-inoculated upper leaves of alfalfa plants by WB with PeSV antiserum and by TEM (Supplementary Material Fig. S3). STD-RT-PCR performed with leaves collected from inoculated alfalfa plants was positive (not shown).

In conclusion, our results indicate that previously reported (Nemchinov et al. 2015) nucleotide sequence of the ALV was 99.9% correct. Only nine nucleotides in the reported sequence (GeneBank: KP784454) were different from the infectious clone (Supplementary Material Table S1). These nucleotides did not match between viral sequences determined by primer walking and Illumina RNA-seq and initial preference was given to the experimental RT-PCR approach (Nemchinov et al. 2015). RNA transcripts generated from the full-length cDNA clone of the virus were biologically functional and led to symptomatic infection in pea plants *P. sativum* cv. Lincoln. Virus particles possessing morphological and serological features of ALV were purified from the pea plants

and used to successfully infect alfalfa germplasm Regen SY (Bingham 1991).

To the best of our knowledge, this is the first report describing the development of a complete cDNA clone of the ALV strain of PeSV and its infectivity in the diagnostic (*Pisum sativum* cv Lincoln) and natural (*Medicago sativa*) hosts. Generation of an infectious cDNA clone is an integral part of reverse genetic approach for any RNA virus; it is also a necessary tool for research on virus-host interactions and transient heterologous expression. Regarding this study, construction of the infectious ALV cDNA clone represents an initial step towards development of an ALV-based vector for gene silencing and expression of foreign proteins in alfalfa. To date, a breakthrough virus-induced gene silencing (VIGS) technology (Burch-Smith et al. 2004), available for other plant species has not been implemented in alfalfa research. An overall goal of the work is to introduce VIGS approach for functional genomics studies in alfalfa.

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Compliance with ethical standards

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Conflict of interest The author declares that he has no conflict of interest.

Human and animal rights This research does not include any animal and/or human trials.

Ethical approval The author bears all the ethical responsibilities of this manuscript.

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Figure S1. Development and characterization of the first infectious clone of alfalfa latent virus, a strain *of Pea streak virus*. Lev G. Nemchinov. Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service Beltsville, MD, USA. Email: lev.nemchinov@ars.usda.gov.

Supplementary Figure S1

Full-length ALV cDNA clone in pUC57(Kan) vector (pALV)

Small font: pUC57(Kan) sequence. Red fond: genomic sequence of ALV. Blue font: T7 promoter. Underlined: KpnI restriction site, ATG initiation codon, NotI and SphI restriction sites, respectively.

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   1  tcgcgcgttt cggtgatgac ggtgaaaacc tctgacacat gcagctcccg gagacggtca
  61  cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg tcagcgggtg
 121  ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctgagagtgc
 181  accatatgcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcaggcgcc
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 421  GACTCACTAT AGATAAACAA CCAACACCCC CTCAATCTTT TTAAAATAAT CCTAAGATCA
 481  TTTTAAAAAC ATCTAAATGG CACTCACTTA TCGTAGTCCA TTGGAGGAAG TGTTGGGTTC
 541  CTTTTCTTCT TCAGAGCAGA GCTTGATCGC GATCCCAGCC ATAGCGAACT TAAAAGGAGT
 601  AGAACAAGAA AACCACAGTC TATTCAATTA TGCGCTGAGC CCAATAGCAA AGCAAAAATT
 661  GATTAGTAAT GGTTTGTATC TCAGTCCTTT CTCAGCTATG CCTCACTCGC ATCCTGTTTG
 721  TAAAACCCTG GAGAATTATT TACTCTACAA AGTCCTACCC AATTATTTAG ATCACAGGTT
 781  TTACTTCGTG GGGATAAAAG AAAGCAAAAT CAATTTCTTG AGGAGTAGAG AGAAGAAATT
 841  AAACCTGTGC GAGGTCGTAA ATAGGTACGT GACCAGTGCT GACAAAGCTC GGTATGGTTC
 901  AGAATTCACT ATCAGGAGGA CCCTGGATAG TGATAATGAG ATTAAATCAA AGTATGGGCG
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1081  GTGCAACCCT ATCAAGATGT TGGCAACTGT TGTAATACCA CCAGAATTAC TTATTGGTGC
1141  TACCGAATCA ATGAACAAAT GGTGTTACAC ATTTAACGTT GAGGATGATG AATTGTTCTT
1201  CTTTCCGGAT GGGGTAACTA CTGAGGGTTA TTCCCAAAGA GCGGATTGTG CTGATCTACT
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1381  GACATTCTCA AATTTTGAAG CTGTGGGCAC AAAAGGGATT GCGGATTTGA TGGAGGGTAA
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1501  TCTCAACAAG CCAGATGTCC AATCTGCCAT TGCAAAGCTA GGACAGATCG TGCAAGAGCC
1561  CAGCGCTTTC GAGATAAAGT TTGTCAGAGA GTACGCCGCA TTGCACATCA GCAAGCGAGG
1621  ATTGAATAGT CTGATATTGA GGGATAATTG GGCAAGAGTC AAAGCTGAAT TCTTGAAACT
1681  CCTCCCTAAA GCTTTTTATG GAAGGTTTGA CACTATCTTA AAGGTAGCCT TGGAGGATTT
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2161  GGCTGAGGAG CAGATTCTGG ATAGAAAGCT ACTCACACAC TTGGAGTTGA GCGTTGAAGT
2221  TAAAGCTGAT CCGGAAAAGA GTGATGAGGA GGAGGTGGAG GAAGTCGGAG AGGAAAAGCC
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2281  TTTTGAAAGG GCTGTCCACC ATATCGATTG CGAATGTGGC ATGAAGTTTG AATGCGGGAA
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2881  GCATGAGGTT GAGATCAAGC GTGGAAGAGA TGCGGATAAG CAGGCTTTAG AAGTGATTGA
2941  AGTGCCTGGT GATGGAAACT GCTTCTGGCA CTCATTGGGT TACTACTTTG GGCTAGAGGG
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3301  TCTTCAAGAC ATCCATAGAT GTCTCACCAA AGCTGAGAAT CGCCACCTCC TTGAACTCGT
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6961  GATCCAATGC CATTGATAGC ACCTCCTAAT AATTCTAATT CTTATCTAGC TCTGGCTATC
7021  GGTGCTGGTT TTGCAATCAT CATCTTCACC CTTAGATCCA ATCAATTGCC TCACGTGGGT
7081  GATAACATTC ATTCATTGCC TCACGGAGGT TTTTACAGAG ACGGGACAAA GGTCATACAA
7141  TATAATTCCC CTGTGAGGAC GCCAAACAAC TGGTTCAAGG GACCTAACAA CATTCAGGCT
7201  TTAGCCCTGG TTTTGTTGGT TATTGGTCTG ATTCATGCGT CATCTGTAAA GATAAGTCGG
7261  GGATGCAGTT GTTCCAAGTA ATCCTAAGTG TGTTTTCCTG CTTTGCGGTT TTGTTGTGCT
7321  TGTATACTAT AGATTCGTTT TTAAATAATT CTGTGTGCCA GTGCACTGTG GTGTTAACTG
7381  GTGAAAGTGT CAAAATTGTC GGGTGTGAAT TTACAAGTGA ATTCATTGAG TACGCCAAAA
7441  CTTTAAAGGT GCAGGCAATT TGAgtatctt taagtttgca gagagtgtat tcgataaagA
7501  TGGCAGAGCA ACAGAAAGCA GCTGAGGAGA GGGCTCGCAT AGATGCGAAG TTGAAGCAGC
7561  AGCTGGAGCA GCAGAGTTCC AAACTGGGAG CTGAGAAAGA GTTTATTAAG GATGCTGAGG
7621  GTCTACAAAA ATCTTTACTT GAGCGGTTCC AGGCCTTACA AGACCTCACC GCTAAAAGCA
7681  TTGCAGGTTC TTCCATCGTT AATGGTGGTT GGGAAGCGGA TCGGAAAAGA CTCAAAGTTT
7741  CTGACAAAAT GAAGCTAGAT GGTTCCAACA TTTTCACAAG GCCAACATTG GACGATCTGC
7801  AGAAGCTGGG CTGGAATCCT GAGGCCAACC AGGTGGCTAC TGCTGAGAAT CTGGCTAAAA
7861  TCAGTGCGAA ATTGAAGGAA TTGGGGGTCC CGCCTGAACA GATGGCAAGG ACTTTTTGGG
7921  ATGTGGCTAT GTATTGTACT GCTGTGGGTG CTTCCAGGTA CACTAATCCA CAAGGGTCAA
7981  TTAACTATCC TGAAGGTTCA ATAACTAGAG ATGCGGTCTT TGGAGTCATT CGAGAGCAAT
8041  GCACTCTGAG GCAGGTTTGC AGGAGCTTCG CTCCTATCAT CTGGAACTAC ATGCATGTTA
8101  ATAATATGCC CCCAACTAAT TGGGCGGAAA AGGGTTTCAG CAATGATGTC AAATTTGCTG
8161  CTTTTGATTT CTTTGATTTC GTGGAGTGCC CAGCTTCAAT TCAACCGGCG GATGGTCTGA
8221  TTAGACGTCC TACATCTGAT GAGTATGTTG CGTTCAACAC TCACAAAAAG TGTGCCCTGG
8281  CCAGGGCTGA CAAGAATGGT CGTTATGCGA GTACTGATGC CTCTGTTACT GGAGGAATGT
8341  TCGGTTGTGG AGCCAAAGAG ACTTGGCGTA ATAATGCGTG CTGAATGTGT GTGCGGTTGC
8401  TAGTAAAACC TAAATAATGT ATAAGCTAGA ACCTATAAAA TCAAGTGTGT TTTAAAATAT
8461  TTTTAATGCT TGTAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAGCGGCCg catgcaagct
```

Figure S1. Development and characterization of the first infectious clone of alfalfa latent virus, a strain *of Pea streak virus*. Lev G. Nemchinov. Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service Beltsville, MD, USA. Email: lev.nemchinov@ars.usda.gov.

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 8521  tggtgtaatc atggtcatag ctgtttcctg tgtgaaattg ttatccgctc acaattccac
 8581  acaacatacg agccggaagc ataaagtgta aagcctgggg tgcctaatga gtgagctaac
 8641  tcacattaat tgcgttgcgc tcactgcccg ctttccagtc gggaaacctg tcgtgccagc
 8701  tgcattaatg aatcggccaa cgcgcgggga gaggcggttt gcgtattggg cgctcttccg
 8761  cttcctcgct cactgactcg ctgcgctcgg tcgttcggct gcggcgagcg gtatcagctc
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 8881  gagcaaaagg ccagcaaaag gccaggaacc gtaaaaaggc cgcgttgctg gcgtttttcc
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 9541  tttctacggg gtctgacgct cagtggaacg aaaactcacg ttaagggatt ttggtcatga
 9601  gattatcaaa aaggatcttc acctagatcc ttttaaatta aaaatgaagt tttaaatcaa
 9661  gcccaatctg aataatgtta caaccaatta accaattctg attagaaaaa ctcatcgagc
 9721  atcaaatgaa actgcaattt attcatatca ggattatcaa taccatattt ttgaaaaagc
 9781  cgtttctgta atgaaggaga aaactcaccg aggcagttcc ataggatggc aagatcctgg
 9841  tatcggtctg cgattccgac tcgtccaaca tcaatacaac ctattaattt cccctcgtca
 9901  aaaataaggt tatcaagtga gaaatcacca tgagtgacga ctgaatccgg tgagaatggc
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10021  aaatcactcg catcaaccaa accgttattc attcgtgatt gcgcctgagc gagacgaaat
10081  acgcgatcgc tgttaaaagg acaattacaa acaggaatcg aatgcaaccg gcgcaggaac
10141  actgccagcg catcaacaat attttcacct gaatcaggat attcttctaa tacctggaat
10201  gctgtttttc cggggatcgc agtggtgagt aaccatgcat catcaggagt acggataaaa
10261  tgcttgatgg tcggaagagg cataaattcc gtcagccagt ttagtctgac catctcatct
10321  gtaacatcat tggcaacgct acctttgcca tgtttcagaa acaactctgg cgcatcgggc
10381  ttcccataca agcgatagat tgtcgcacct gattgcccga cattatcgcg agcccattta
10441  tacccatata aatcagcatc catgttggaa tttaatcgcg gcctcgacgt ttcccgttga
10501  atatggctca taacacccct tgtattactg tttatgtaag cagacagttt tattgttcat
10561  gatgatatat ttttatcttg tgcaatgtaa catcagagat tttgagacac gggccagagc
10621  tgca
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Development and characterization of the first infectious clone of alfalfa latent virus, a strain *of Pea streak virus*.
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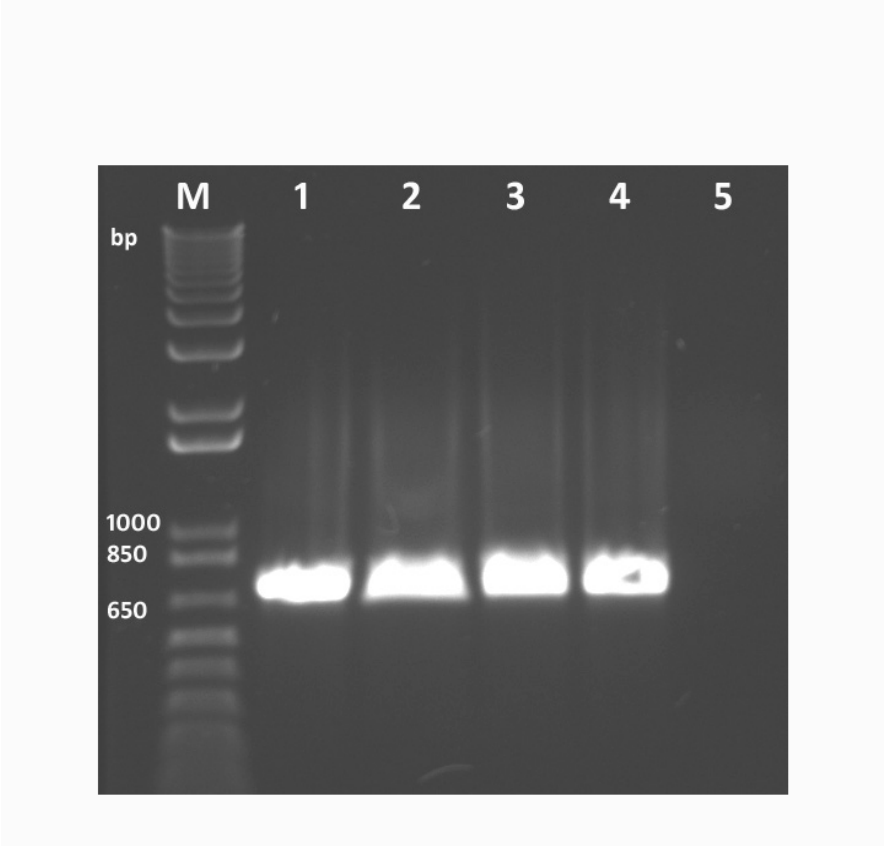
Supplementary Figure S2



PCR products amplified from purified ALV preparations using STD-RT-PCR protocol (Suehiro et al., 2005). The virus was purified from transcripts-inoculated plants. M, 1 Kb Plus DNA Ladder (Thermo Fisher Scientific). Lanes 1- 4: PCR products amplified from purified ALV preparations. Lane 5: water control.

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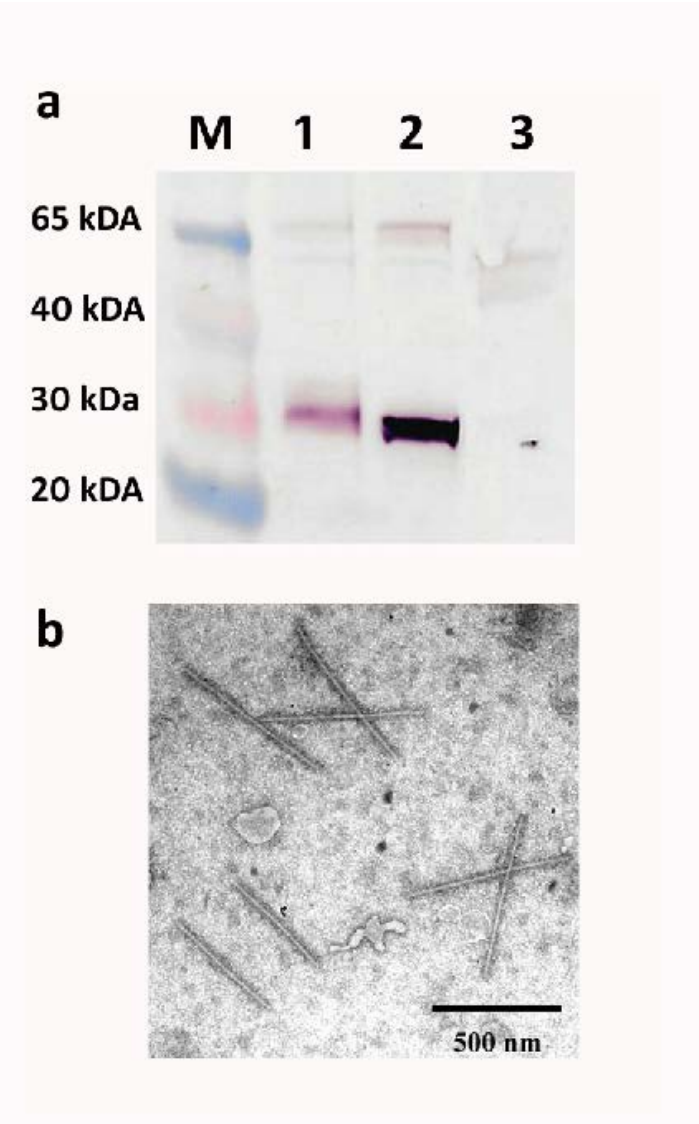
Supplementary Figure S3



Serological and microscopic detection of ALV in crude extracts of alfalfa germplasm Regen SY inoculated with purified viral preparations from pea *P. sativum* cv. Lincoln. a Western blot assay using polyclonal antiserum to PeSV. M, Color Burst electrophoresis marker (Sigma-Aldrich). Lanes 1-3: ALV positive control, infected alfalfa plant Regen SY and uninfected alfalfa control, respectively. **b** TEM micrograph of ALV particles from infected alfalfa. Scale bar represents 500 nm.

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Supplementary Table S1

Nucleotide differences between the reported ALV genome (GeneBank: KP784454) and the full-length infectious cDNA clone (this work)

GeneBank: KP784454 → infectious cDNA clone	**Silent/missense**
A→G (nt2605)	GAA(Glu)→GAG(Glu), silent
C→T (nt3439)	CTC(Leu)→CTT(Leu), silent
T→C (nt3742)	GGT(Gly)→GGC(Gly), silent
C→A (nt6530)	GGC(Gly)→GGA(Gly), silent
A→T (nt6531)	ACA(Thr)→TCC(Ser), missense
A→C (nt6533)	
C→A (nt6534)	CAT(His)→AAT(Asn), missense
T→C (nt6869)	TGT(Cys)→TGC(Cys), sillent
G→T (nt6871)	TGT (Cys)→TTT(Phe), missense