

Lymantria dispar iflavirus 1 (LdIV1), a new model to study iflaviral persistence in lepidopterans

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The cell line IPLB-LD-652Y, derived from the gypsy moth (*Lymantria dispar* L.), is routinely used to study interactions between viruses and insect hosts. Here we report the full genome sequence and biological characteristics of a small RNA virus, designated *Lymantria dispar* iflavirus 1 (LdIV1), that was discovered to persistently infect IPLB-LD-652Y. LdIV1 belongs to the genus *Iflavirus*. LdIV1 formed icosahedral particles of approx. 30 nm in diameter and contained a 10 044 nt polyadenylated, positive-sense RNA genome encoding a predicted polyprotein of 2980 aa. LdIV1 was induced by a viral suppressor of RNA silencing, suggesting that acute infection is restricted by RNA interference (RNAi). We detected LdIV1 in all tested tissues of gypsy-moth larvae and adults, but the virus was absent from other *L. dispar*-derived cell lines. We confirmed LdIV1 infectivity in two of these cell lines (IPLB-LD-652 and IPLB-LdFB). Our results provide a novel system to explore persistent infections in lepidopterans and a new model for the study of iflaviruses, a rapidly expanding group of viruses, many of which covertly infect their hosts.

Received 8 May 2014
Accepted 26 June 2014

INTRODUCTION

The development of tissue- and cell-culture systems was initiated more than a century ago; since then, these technologies have become indispensable tools for studies in the life sciences under controlled conditions (Alberts *et al.*, 2002). In the field of virology, primary or established cell lines have been essential for understanding the molecular basis of infection cycles, identification of viral and host factors involved in resistance and susceptibility, the discovery of antiviral drugs, the production of vaccines and the manufacture of viral and heterologous proteins, among many other applications (Drugmand *et al.*, 2012; Lynn, 2001).

The cell line IPLB-LD-652Y (Goodwin *et al.*, 1978), derived from the gypsy moth (*Lymantria dispar* L.), has been used as a model for virus–host interactions, especially for baculoviruses (Guzo *et al.*, 1991; Lynn, 2006; McClintock *et al.*, 1986; McIntosh *et al.*, 2005), but also for studies of entomopoxviruses (Winter *et al.*, 1995), polydnviruses (Kim *et al.*, 1996; McKelvey *et al.*, 1996) and some picorna-like viruses (Ongus *et al.*, 2006). We attempted to use the cell line IPLB-LD-652Y to study replication of a honey

bee-infecting RNA virus in the *Dicistroviridae*. Surprisingly, we found a completely different virus of similar shape and size in the negative-control cultures that had not been transfected with the dicistrovirus. On the basis of morphological and genomic features, this virus, designated *Lymantria dispar* iflavirus 1 (LdIV1), appears to be the first member of a new species of the genus *Iflavirus* (family *Iflaviridae*).

Iflaviruses form icosahedral non-enveloped particles and have a positive-sense RNA genome. They infect invertebrates, primarily insects (Table 1). The structural proteins are encoded on the 5' half of the genome and the non-structural proteins on the 3' half (Chen *et al.*, 2012b; Hulo *et al.*, 2011). According to the International Committee on Taxonomy of Viruses (ICTV), *Iflavirus* is the sole genus in the recently recognized family *Iflaviridae*, and its species are demarcated by host range and <90% aa identity in the sequence of the capsid-protein precursor (Chen *et al.*, 2012b; Kuhn & Jahrling, 2010); LdIV1 fulfils all of the requirements for a new species. Currently, 18 full-length genomes of iflaviruses (Table 1) are in the GenBank database at the National Center for Biotechnology Information (NCBI), along with some partial sequences of potential iflaviruses (He *et al.*, 2013; Oliveira *et al.*, 2010; Reineke & Asgari, 2005). Many iflaviruses infect their host without inducing disease signs in a persistent manner and are transmitted vertically *in vivo*, all of which are characteristics

The GenBank/EMBL/DDBJ accession number for the genome sequence of *Lymantria dispar* iflavirus 1 is KJ629170.

Four supplementary figures, three supplementary tables and Supplementary Methods are available with the online version of this paper.

Table 1. Members of *Iflaviridae* with full-length genomes in GenBank and identity to LdIV1 at the aa level

The first isolate of each species listed was used for the analyses in this work. NA, Not applicable.

Name	Acronym	Genome size (nt) without polyA	Polyprotein ORF nt coordinates (total no. of aa)	NCBI genome accession nos. of genome/polyprotein	% identity (% similarity) to LdIV1 polyprotein	Host common name/class/order	Reference
Lymantria dispar iflavirus 1	LdIV1	10 044	937–9879 (2980)	KJ629170	NA	Moths/Insecta/Lepidoptera	This work
Infectious flacherie virus*	IFV	9650	157–9414 (3086)	NC_003781/NP_620559	17.9 (34.4)	Moths/Insecta/Lepidoptera	Isawa <i>et al.</i> (1998)
Sacbrood virus – Rothamstead	SBV-Roth	8832	179–8752 (2858)	NC_002066/NP_049374	20.6 (37.1)	Honey bee/Insecta/Hymenoptera	Ghosh <i>et al.</i> (1999)
Perina nuda virus	PnV	9476	474–9431 (2986)	NC_003113/NP_277061	18.3 (35.2)	Moths/Insecta/Lepidoptera	Wu <i>et al.</i> (2002)
<i>Ectropis obliqua</i> virus*	EoV	9394	391–9351 (2987)	NC_005092/NP_919029	18.2 (35.0)	Moths/Insecta/Lepidoptera	Wang <i>et al.</i> (2004)
Varroa destructor virus 1 – Netherlands*	VDV-1-NL	10 112	1118–9799 (2893)	NC_006494/YP_145791	30.4 (48.2)	Mites/Arachnida/Parasitiformes	Ongus <i>et al.</i> (2004)
Deformed wing virus – Italy*	DWV-IT	10 135	1140–9821 (2894)	NC_004830/N_P_853560	30.5 (47.9)	Honey bee/Insecta/Hymenoptera	Lanzi <i>et al.</i> (2006)
Brevicoryne brassicae virus	BrBV	10 161	793–9744 (2983)	NC_009530/YP_001285409	26.8 (44.7)	Aphids/Insecta/Hemiptera	Ryabov (2007)
Slow bee paralysis virus	SBPV	9482	317–9208 (2964)	NC_014137/YP_003622540	26.4 (43.5)	Honey bee/Insecta/Hymenoptera	de Miranda <i>et al.</i> (2010)
Spodoptera exigua iflavirus 1	SeIV-1	10 347	345–10 010 (3223)	NC_016405/YP_004935363	17.8 (33.2)	Moths/Insecta/Lepidoptera	Millán-Leiva <i>et al.</i> (2012)
Spodoptera exigua iflavirus 2	SeIV-2	9501	392–9421 (3011)	JN870848/AFQ98017	18.1 (35.6)	Moths/Insecta/Lepidoptera	Choi <i>et al.</i> (2012)
Lygus lineolaris virus 1	LyLV-1	9635	604–9564 (2986)	JF720348/AEL30247	19.8 (35.2)	Tarnished plant bug/Insecta/Hemiptera	Perera <i>et al.</i> (2012)
Nilaparvata lugens honeydew virus-1	NLHV-1	10 937	1137–10 664 (3175)	AB766259/BAN19725	25.2 (42.1)	Brown planthopper/Insecta/Hemiptera	Murakami <i>et al.</i> (2013)
<i>Nilaparvata lugens</i> honeydew virus-2	NLHV-2	10 985	989–10 726 (3245)	NC_021566/YP_008130309	23.8 (40.6)	Brown planthopper/Insecta/Hemiptera	Murakami <i>et al.</i> (2014)
Nilaparvata lugens honeydew virus-3	NLHV-3	10 600	784–10 311 (3175)	NC_021567/YP_008130310	26.8 (43.7)	Brown planthopper/Insecta/Hemiptera	Murakami <i>et al.</i> (2014)
Formica exsecta virus 2 – Fex2	†	9139	165–8895 (2910)	KF500002/AHB62422	28.8 (46.4)	Ants/Insecta/Hymenoptera	Johansson <i>et al.</i> (2013)
Halyomorpha halys virus – Beltsville	†	9271	152–9202 (3016)	NC_022611/YP_008719809	20.0 (36.2)	Stink bug/Insecta/Hemiptera	Sparks <i>et al.</i> (2013)
Antheraea pernyi iflavirus – LnApIV-02	ApIV	10 163	884–9994 (3036)	KF751885/AHI87751	70.8 (83.1)	Moths/Insecta/Lepidoptera	Geng <i>et al.</i> (2014)
Heliconius erato iflavirus	†	9910	906–9803 (2965)	KJ679438/AHW98099	64.4 (79.2)	Butterflies/Insecta/Lepidoptera	Smith <i>et al.</i> (2014)

*Members of the genus *Iflavirus* recognized by the ICTV (Chen *et al.*, 2012b).

†Original reports did not suggest abbreviations for these viruses.

of covert, persistent or chronic infections (de Miranda & Genersch, 2010).

In this paper, we report the characteristics of LdIV1 and discuss the potential for its use as a biocontrol tool for the gypsy moth, a serious forest pest introduced accidentally into North America (Moore, 2009). In addition, we show that a viral suppressor of RNA silencing (Nayak *et al.*, 2010) increases the accumulation of LdIV1, and we propose that this strategy can be used as a diagnostic tool for discovering covert and persistent infections in apparently virus-free cell lines.

RESULTS

Discovery of a spherical RNA virus with iflavivirus characteristics in IPLB-LD-652Y cells

While attempting to infect the cell line IPLB-LD-652Y with a honey bee-infecting dicistrovirus, we detected icosahedral

virions of approximately 30 nm in diameter in the cells by transmission electron microscopy (TEM). Although these particles were of the expected size and shape for a dicistrovirus, we were surprised to find virions in cells transfected with non-infectious transcripts and also in untreated cells (Fig. 1a and Fig. S1, available in the online Supplementary Material). Because our work routinely involves manipulation of several picorna-like viruses from the honey bee and from aphids, we first ran an retro-transcription-PCR (RT-PCR) screen using diagnostic primers for each virus to eliminate the possibility of contamination (Table S1 and Table S2). All of these PCR screens yielded no amplification (data not shown). We proceeded with the cloning and characterization of the unknown virus. An initial PCR amplification with degenerate primers designed to amplify conserved picornavirus sequences (Table S1) resulted in amplification products whose sequences did not share significant identity with nt sequences from other virus sequences in GenBank as determined by BLASTN. However,

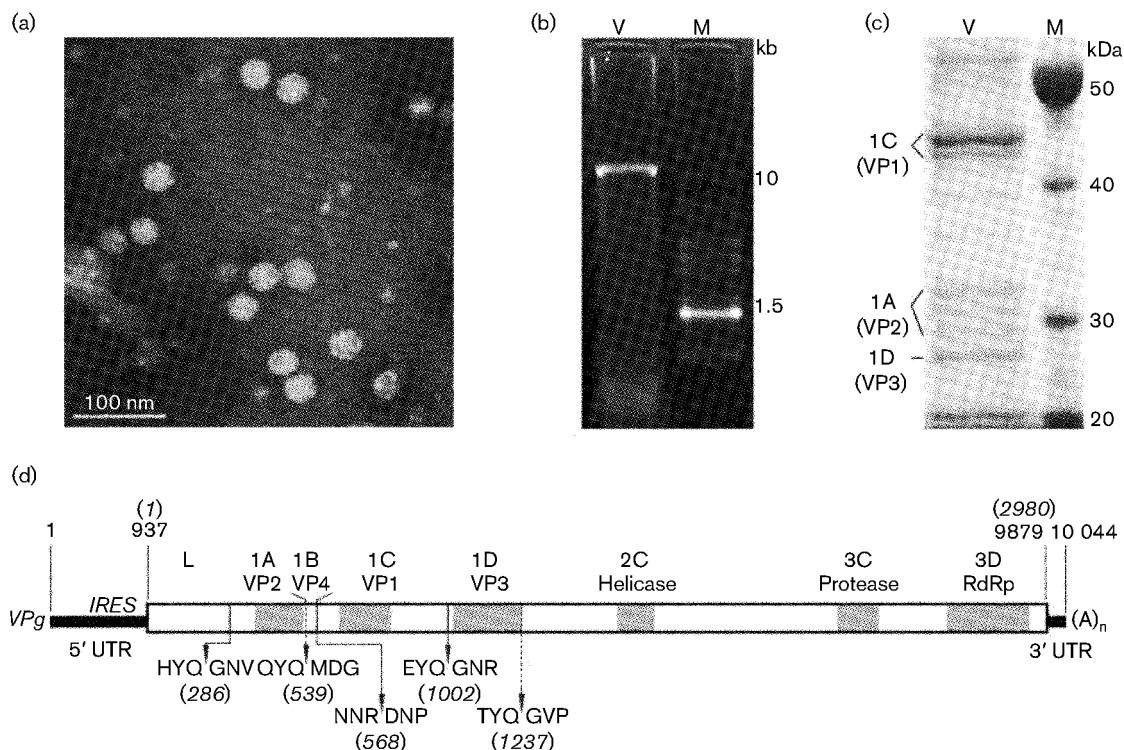


Fig. 1. Characteristics of LdIV1. (a) Transmission electron micrograph of virus particles obtained from IPLB-LD-652Y cells showing extensive CPE. (b) Viral RNA analysed by electrophoresis in 1.2% denaturing agarose gel stained with ethidium bromide. V, total RNA extracted from semi-pure LdIV1 particles; M, RNA-size marker indicating the size of two reference bands. (c) SDS-PAGE of LdIV1 virions showing the major structural proteins (virion proteins, VP) identified by Edman sequencing. V, total proteins after semi-purification of virus particles; M, protein marker indicating masses of reference bands. (d) Schematic illustration of the LdIV1 genome showing the positions of viral proteins and non-coding elements. Numbers in plain text indicate nt coordinates and numbers in italics in parentheses indicate aa coordinates. Boxes in 1A and 1C, picorna-like capsid drug-binding pocket domain; box in 1D, CrPV capsid-protein-like domain; box in 2C, helicase domain; box in 3C, protease domain; box in 3D, RdRP domain. Predicted features, including viral protein genome-linked (VPg) to 5' end and IRES presence in 5' UTR, are shown in italics. Arrows indicate cleavage sites in the shown aa sequences and positions identified by Edman degradation (solid lines) or that are predicted (dashed line).

BLASTX indicated that the amplified sequences displayed low sequence identity to iflavirus aa sequences. One group of clones had significant sequence similarity to the iflaviral coat-protein precursor and another group matched an RNA-dependent RNA polymerase (RdRP) region.

Particles and viral genome

Viral RNA was extracted from virions that were partially purified from untreated cells and analysed by denaturing agarose gel electrophoresis. A single band of approx. 10 kb was observed, and no subgenomic viral RNAs were detected (Fig. 1b). This RNA was used as a template to amplify cDNA fragments to sequence the full viral genome using the initial amplicons as starting points. The termini of the genome were determined by 5' and 3' RACE as described in Methods. We named the virus LdIV1, and on the basis of its characteristics, we propose that LdIV1 represents a new species in *Iflavirus*, which fits the genus description: a genome of 10 044 nt (not including the poly A tail) comprising a 937 nt 5' untranslated region (UTR) with a putative internal ribosome entry site (IRES), a single ORF

predicted to encode a polyprotein of 2980 aa (Table 1) and a 165 nt 3' UTR followed by a poly A tail (Fig. 1d). The nt composition of the LdIV1 sequence was G, 19.73 mol%; A, 31.21 mol%; U, 33.76 mol%; C, 15.15 mol%; and N, 0.14 mol% [representing variants of the genome quasispecies (Domingo *et al.*, 2012)]. A BLASTX analysis of the LdIV1 genome returned top hits to *Antheraea pernyi* iflavirus (ApIV), *Heliconius erato* iflavirus, deformed wing virus (DWV) and *Varroa destructor* virus-1 (VDV-1), all of which are members of *Iflaviridae*. A phylogenetic tree reconstructed after alignment of the full-length polyproteins from reported iflaviruses grouped LdIV1 with ApIV in a branch close to the DWV/VDV-1 cluster (Fig. 2).

5' UTR and polyprotein start codon

The reported 5' UTRs of iflaviruses vary enormously in length (from 152 to 1140 nt; see polyprotein coordinates in Table 1). Because they likely contain IRESs, these UTRs often contain many AUG triplets that are not start codons. A number of putative start codons in frame with the predicted polyprotein were identified in the LdIV1 genome, including

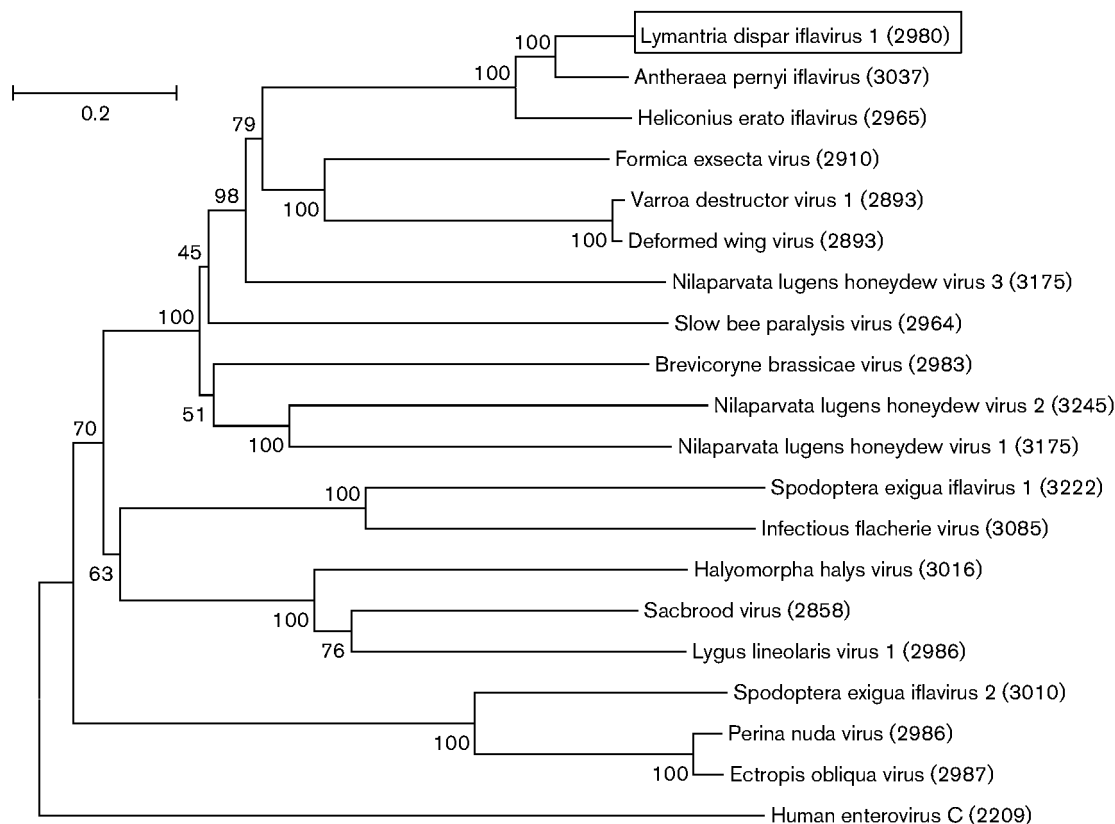


Fig. 2. Phylogenetic relationships of LdIV1 with other iflaviruses. Full-length polyprotein sequences were used (see Table 1). The tree was reconstructed with the neighbour-joining method and evaluated with bootstrap analysis (1000 replicates; percentage support is shown for every branch). Branch lengths represent the number of aa substitutions per site (scale bar indicates evolutionary distance for 0.2 aa substitutions per site). Human enterovirus C (NP_041277) was included as an outgroup. The length of each polyprotein (in aa) is shown in parentheses.

AUG codons at coordinates 937 (AAGAUGGC) and 571 (GUUAUGG) that follow Kozak's rule for a good start-codon context (consensus sequence RNNAUGG) in eukaryotes (Kozak, 1999), but AUGs at coordinates 382 (AGUAUGA) and 937 (AAGAUGG) fit sequences frequently found in invertebrate start codons [ANNAUG(A/G)C] (Cavener & Ray, 1991). More experimental work is needed to corroborate or determine the precise polyprotein-starting point, since according to reported ORFs of other iflaviruses, the Kozak and invertebrate consensus sequences may not be required for efficient translation initiation because of the presence of IRESs (Lu *et al.*, 2007; Ongus *et al.*, 2006). A highly stable secondary structure (936 nt of 5' UTR; $\Delta G = -237.5$) was predicted using MFOLD software (Zuker, 2003), indicating the possible presence of an IRES. From the above considerations, we propose that LdIV1 has a 5' UTR of 936 nt. It is possible, however, that the 5' end identified by the 5' RACE is not definitive, as a strong secondary structure could impede acquisition of the true 5' end (Murakami *et al.*, 2013), and more than one 5' end may be present among viral variants (Murakami *et al.*, 2013, 2014).

Polyprotein sequence analysis

In this paper, we follow the L434 nomenclature for picornaviral proteins (Rueckert & Wimmer, 1984) as first adopted for iflaviruses by Murakami *et al.* (2013). This system allows for naming of proteins based on genome position and sequence identity rather than on size or molecular mass. Nevertheless, in Fig. 1(d), we included the names of structural proteins (i.e. capsid or viral proteins), following the nomenclature of the iflavirus type species, infectious flacherie virus (Chen *et al.*, 2012b; Isawa *et al.*, 1998). Using BLASTP and Conserved Domain Database (CDD) tools (Altschul *et al.*, 1997, 2005; Marchler-Bauer *et al.*, 2011, 2013), we found two picornavirus capsid domains, including drug-binding pockets in 1A (aa 368–523) and in 1C (aa 648–812); a cricket paralysis virus (CrPV) capsid-like domain in 1D (aa 1019–1246); an RNA helicase domain (comprising motifs A, B and C at aa 1562–1675); and an RdRP (from motif I to VIII at aa 2649–2913). LdIV1 helicase, protease and RdRP domains matched corresponding conserved regions reported for positive-strand RNA viruses (Koonin *et al.*, 1993). A 3C-like protease domain was not detected with the CDD tool but was identified by comparison with other iflaviruses and picornavirid protease domains (de Miranda & Genersch, 2010; Gorbalenya *et al.*, 1989; Ryan & Flint, 1997; Ye *et al.*, 2012). The 3C domain includes a proposed catalytic triad H₂₂₉₄, D₂₃₂₁, C₂₄₀₃; a cysteine protease motif ₂₄₀₁GXCG₂₄₀₄; and a substrate-binding site ₂₄₁₈GxHxxG₂₄₂₃ (Gorbalenya *et al.*, 1989). A leader protein is expected at the N terminus of the polyprotein on the basis of the ORF prediction and the sequence of N termini of capsid proteins (see below).

Structural proteins

When semi-pure viral particles were denatured and analysed by SDS-PAGE, we sometimes noted that the viral structural

proteins (expected to be in the 25–45 kDa range, with the exception of 1B) ran as doublets or triplets (Fig. 1c). Sequencing of these bands by N-terminal Edman degradation (Table S3) revealed that protein 1A migrated as three clearly separate bands and protein 1C as two bands. These results suggest that the structural proteins 1A and 1C could have variable C termini, as has been suggested for other iflavirus coat proteins (de Miranda *et al.*, 2010). Alternatively, the multiple-banding pattern may reflect post-translational modifications or an association of these proteins with cellular proteins not released by either the virion-enrichment protocol or the denaturing treatment before gel loading. Cleavage sites for LdIV1 structural proteins followed the Q/G rule for 3C-pro picornaviral proteases (Blom *et al.*, 1996; Isawa *et al.*, 1998), except at the 1B–1C junction where an NR/D cleavage site was found, in agreement with the cleavage sequence at this site observed in other iflaviruses (Murakami *et al.*, 2013). Cleavage at the C terminus of 1D was predicted using the NetPicoRNA server (Blom *et al.*, 1996) (Fig. 1d). On the basis of these cleavage sites, the predicted molecular masses for 1C, 1D and 1A+1B were 48.2 kDa, 26.7 kDa and 31.4 kDa, respectively. We were unable to detect virion protein 1B by PAGE; this protein is the smallest structural protein and is predicted to be around 2 kDa in DWV and in VDV-1 (de Miranda & Genersch, 2010). The cleavage site between 1A and 1B is not well conserved in this family. In the LdIV1 structural protein precursor, the region between the drug-binding pocket of 1A and the identified N terminus of 1C (aa 524–567) includes a Q/M site that could correspond to the 1A/1B cleavage site predicted by NetPicoRNA and by sequence identity with ApIV (Fig. 1d). The estimated masses for 1A and 1B would be 28.4 kDa and 2.9 kDa, respectively, in agreement with the pattern observed in SDS-PAGE (Fig. 1c).

LdIV1 infects IPLB-LD-652Y cells in a covert, persistent fashion

For our dicistrovirus work, we performed various transfections with fluorescent markers (mCherry and eGFP) to check transfection efficiencies (Fig. S2a). To increase dicistroviral replication, we also used a heterologous, strong viral suppressor of RNAi from CrPV (CrPV-1A VSR) (Nayak *et al.*, 2010). We observed that cells transfected with these treatments in the absence of the dicistrovirus showed different CPEs (Fig. S2b). We noted that cells transfected with CrPV-1A showed more damage than cells in the other treatments (Fig. 3a), and that virus particles were easily detected (Fig. S1). We measured LdIV1 loads in these cells until 5 days after treatment, and in all cases, the LdIV1 titre [as determined by real-time RT-PCR (RT-qPCR) amplification of viral genome (coordinates, 1716–1830)] increased with the age of the culture. In contrast, in cells transfected with CrPV-1A VSR, a rise in the amount of viral genome was evident as early as 2 days post-treatment (p.t.) (Fig. 3b), suggesting that the RNAi pathway is involved in suppression of LdIV1.

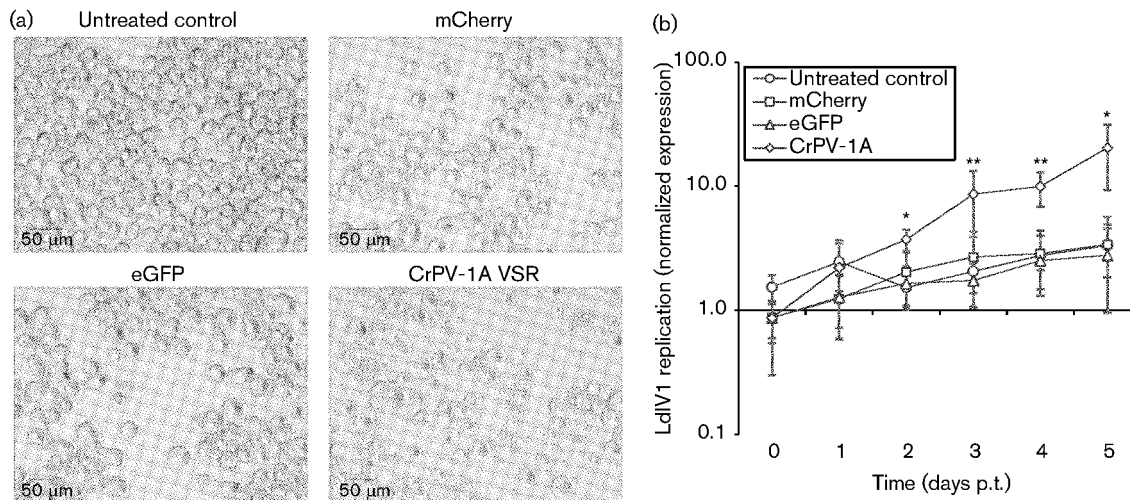


Fig. 3. LdIV1 replication in IPLB-LD-652Y cells. (a) Representative pictures of cell cultures transfected with two fluorescent markers (mCherry and eGFP), CrPV-1A VSR and untransfected cells (as a control) at 2 days p.t. (b) LdIV1 relative quantification in total RNA (100 ng) from cells of the different transfections sampled at 0–5 days p.t. Time zero was taken as calibrator (equal to 1); for relative quantification, LdIV1 amounts were normalized to expression of two internal reference genes. Results from one representative experiment are shown. Asterisks indicate statistically significant differences when viral loads were compared among all treatments at the same time point (one-way ANOVA, * $P \leq 0.001$, ** $P < 0.0005$; $N = 3$; error bars represent 95% confidence intervals).

Presence of LdIV1 in different tissues and developmental stages of the gypsy moth

We used RT-PCR with two sets of primers for detection of LdIV1 sequences in RNA harvested from eggs and tissues from larvae and adults of the gypsy moth. LdIV1 was detected in every tissue and developmental stage examined (Fig. 4a). The presence of LdIV1 in fat-body and ovariole tissues was not consistent with the fact that a fat-body-derived cell line (IPLB-LdFB) and some ovary-derived cells (IPLB-LD-65 and IPLB-LD-652) were virus free (see below). It is important to note that material for the tissue examination was pooled from several individuals (see Supplementary Methods). Screening of individual specimens is needed to study the presence of LdIV1 in different tissues at the individual level as well as the viral incidence at the population level.

Testing LdIV1 infectivity in other cell lines

The presence of infectious virus in apparently healthy insects (and cells) is consistent with a covert-persistent infection (de Miranda & Genersch, 2010). We were able to detect LdIV1 in gypsy moths and also found viral particles in untreated IPLB-LD-652Y cells (Fig. S1). We next looked for LdIV1-free lepidopteran cell lines to test for viral infectivity of LdIV1 particles to fulfil the Koch/Rivers postulates (Rivers, 1937). We screened other cell lines derived from the gypsy moth and from the fall armyworm (*Spodoptera frugiperda*). As shown in Fig. 4(a), LdIV1 RNA was detected in IPLB-LD-652Y stocks from different laboratories (see Methods) and also in the embryonic cell line IPLB-LdEp

(Lynn *et al.*, 1988). In the latter cell line, we were unable to amplify the 5' end (1200 nt) of the LdIV1 genome, perhaps because of the presence of strong RNA secondary structures as discussed above. However, some gypsy-moth cell lines appeared to be LdIV1 free, including IPLB-LdFB, derived from larval fat body (Lynn *et al.*, 1988), IPLB-LD-65 and IPLB-LD-652, derived from ovarian tissues (Goodwin *et al.*, 1978), and fall armyworm cell lines IPLB-SF21 and IPLB-SF9 (Summers & Smith, 1987; Vaughn *et al.*, 1977).

We inoculated IPLB-LdFB, IPLB-LD-652 and IPLB-SF21 by using supernatant from IPLB-LD-652Y cultures and followed LdIV1 replication by RT-qPCR until 10 days after inoculation. IPLB-LdFB and IPLB-LD-652 proved to be suitable hosts for LdIV1 as viral loads clearly increased over time. At 10 days p.t., compared with the amount of viral inoculum originally added, we found >25 and >17 times more LdIV1-genome equivalents in IPLB-LdFB and IPLB-LD-652, respectively (Fig. 4b). For the IPLB-SF21 cell line, an initial decline (likely resulting from degradation of the inoculum) was followed by a gradual increase in virus-genome equivalents, suggesting that the virus could replicate to some extent in this cell line. The severity of CPES correlated with viral titre with disrupted, enlarged, misshapen or vacuolated cells observed after 6 days p.t. (Fig. S3).

DISCUSSION

Original source of LdIV1

In 1978, Goodwin *et al.* reported the establishment of several cell lines from the gypsy moth. These cell lines, derived from

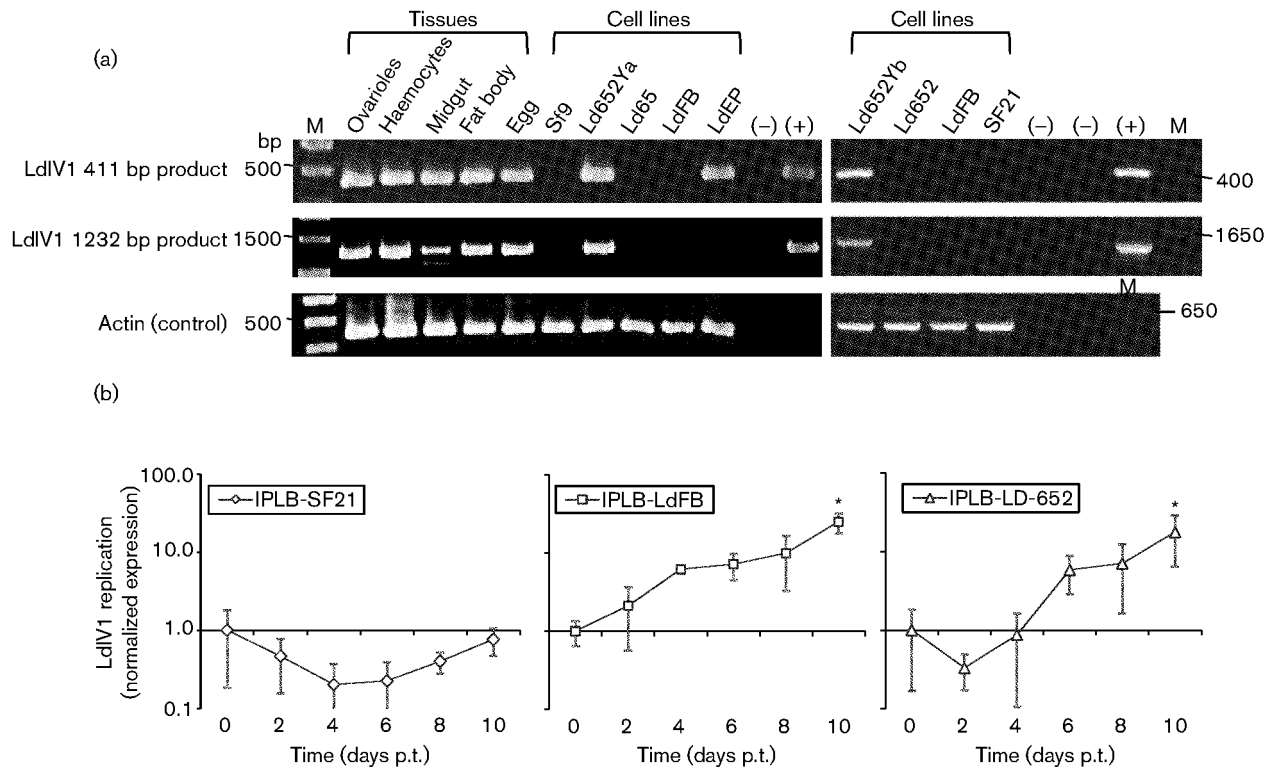


Fig. 4. Detection of LdIV1 in five different *L. dispar* tissues and four different cell lines. (a) RT-PCR products amplified with primers specific for LdIV1 and for the actin gene were visualized by 1.5% agarose electrophoresis and ethidium bromide staining. Expected products from top to bottom: 411 bp, 1232 bp and ~620 bp. M, size marker; -, no template; +, cloned LdIV1 fragment. Sizes (bp) of selected size markers are indicated at the side of each gel. Ld652Ya is cell line maintained in Beltsville and Ld652Yb a cell line maintained in Ames. (b) LdIV1 infectivity in the three lepidopteran cell lines IPLB-SF21, IPLB-LdFB and IPLB-LD-652. IPLB-LD-652Y medium was used as inoculum, and samples were taken at different time points after inoculation. Relative amounts of LdIV1 in 100 ng total RNA were measured by RT-qPCR. Time zero (equal to 1) was used as calibrator for each treatment. One representative experiment is shown. Statistically significant differences in final viral amounts compared with starting inoculum in each cell line are denoted (*t*-test, * $P \leq 0.007$; $N=3$; error bars represent 95% confidence intervals).

pupal ovarian tissue, included the line IPLB-LD-65 and two sublines, IPLB-LD-652 and IPLB-LD-65Y (Goodwin *et al.*, 1978). The line IPLB-LD-652Y was not explicitly reported in this work but has been attributed to these authors thereafter (Guzo *et al.*, 1991; Lynn, 2006; McClintock *et al.*, 1986; McKelvey *et al.*, 1996; Ongus *et al.*, 2006). The IPLB-LD-652Y name may have resulted from a typographical error when referring to IPLB-LD-65Y. Interestingly, we found that the IPLB-LD-65 cell line and its subline IPLB-LD-652 are LdIV1 free (Fig. 4a). Assuming that IPLB-LD-652Y is in fact derived from IPLB-LD-65, there are two possible scenarios to explain the presence of LdIV1. The first is that the parental line IPLB-LD-65 consisted of a mixture of virus-free and -infected cells but that LdIV1 was lost over time. It is well established that cell-culture conditions impose selective pressures that can affect outcomes in virus-host interactions in specific cell lines (Lynn, 2006). Another possibility is that the IPLB-LD-652Y subline was infected with LdIV1 from insects or other cell lines maintained simultaneously in laboratories that have worked with this

cell line. The presence of LdIV1 may have affected the outcomes of research using these infected cell lines.

Mechanism of LdIV1 persistence

The IPLB-LD-652Y cell line needs to be passaged before confluence to maintain healthy-looking cells with similar shape and size, and little to no vacuolation. We observed clear CPEs by transfection with a transcript coding for CrPV-1A (Fig. 3a), a protein that interacts with the endonuclease Ago2 and inhibits the silencing pathway in *Drosophila* (Nayak *et al.*, 2010). The subsequent increase in viral RNA loads (Fig. 3b) likely resulted from inhibition of LdIV1 silencing, suggesting that acute infection with LdIV1 is repressed by RNAi, consistent with previous reports on RNAi-mediated viral persistence (Goic & Saleh, 2012; Jovel & Schneemann, 2011; Nayak *et al.*, 2010). In a recent report, Goic *et al.* demonstrated that persistence of flock house virus and *Drosophila* C virus in *Drosophila* cell lines results from the combined action of retrotransposon-associated

reverse transcriptases and RNAi. In this model system, viral DNA is generated as an intermediate step for establishment of persistence (Goic *et al.*, 2013). Although several retro-transposons have been reported in the gypsy moth (Garner & Slavicek, 1999; Pfeifer *et al.*, 2000), we did not detect LdIV1-DNA elements in cellular DNA (Fig. S4).

Viral persistence in lepidopteran cell cultures

Here, we describe a new virus persistently infecting a cell line that is widely used for studying insect virus–host interactions. Our results provide a warning for virologists using cell cultures that may contain persistent viral infections; such viruses may alter cellular conditions or express *trans*-acting viral proteins that affect replication of challenging viruses leading to misinterpretation of results. Other groups have reported similar findings: Li *et al.* (2007) found an alphanodavirus (Tn5 cell line (TNCL) virus) when trying to use a *Trichopulsia ni* cell line for baculovirus-directed protein expression. As pointed out by the authors, this kind of infection can persist undetected for years because it does not induce overt signs of infection. Other examples of picornavirids that persistently infect cell lines include *Galleria mellonella* cell line virus, discovered in the cell line Gm120, derived from the honeycomb moth when infected with maize stem borer virus (Léry *et al.*, 1997); a nodavirid co-infecting with a parvovirid [not a picornavirid, but a DNA virus (Léry *et al.*, 1998)]; and Lymantria vacuolating virus, a virus originally detected in a different *L. dispar* cell line called SCLd but that could infect IPLB-LD-652Y cells (Kazuhiko *et al.*, 1996). It is possible that some of these viruses belong to *Iflaviridae*, but it was difficult to assess phylogenetic relationships because genome sequences for many of these viruses have not been reported. New techniques for massive sequencing (next-generation sequencing, NGS) will allow for the discovery and rediscovery of many more iflaviruses, as has already occurred in recent years (Table 1). Nonetheless, in some cases, NGS does not always detect covert infections. A recent transcriptome analysis revealed the presence of virus-associated transcripts in the IPLB-LD-652Y cell line (Sparks & Gundersen-Rindal, 2011); however, LdIV1 sequences were not found. In this case, the quantity of viral genomes during the covert infection may have been too low for detection. Our results suggest that viral copies per cell increase as the cell line ages, and that viral genome quantity is relatively low in cells that appear to be healthy and higher in cells that appear to be under stress and exhibiting CPE (Fig. 3). As LdIV1 was activated by CrPV-1A, we propose that this or other suppressors of RNAi could be used to induce acute infections for discovery of persistent virus infections in cell lines.

Application as a biocontrol tool

The gypsy moth is an important pest in the USA since its introduction decades ago and is still considered a dangerous threat to forests (Moore, 2009; Sharov *et al.* 2002). The

original motivation to generate IPLB-LD cell lines was the development of biocontrol tools (Goodwin *et al.*, 1978). The discovery of a covert, persistently infecting virus such as LdIV1 may provide more options for biocontrol strategies. Small RNA viruses have potential as biocontrol tools because of their size and in many cases because of their lethality (Chen *et al.*, 2012a). Viruses that establish persistent infections can be engineered as vectors to express specific toxic proteins or can be modified to increase their virulence. An alternative approach is the administration of viral suppressors of RNA silencing, infection with a second virus or other strategies that could convert persistent, asymptomatic infections to overt, pathogenic infections in pest insects. As far as we know, our work presents the first lepidopteran cell-culture system for exploration of iflaviral persistence and acute infection. Having this model will enable studies of these infection mechanisms at the molecular level to increase our knowledge of virus persistence.

METHODS

Cell lines and insects. Cell lines used in this study include *L. dispar* ovarian cell lines IPLB-LD-65, IPLB-LD-652 and IPLB-LD-652Y (Goodwin *et al.*, 1978); the *L. dispar* embryonic cell line IPLB-LdEp and the fat-body cell line IPLB-LdFB (Lynn *et al.*, 1988); and *S. frugiperda* ovarian cell lines IPLB-SF21 and IPLB-SF9 (Summers & Smith, 1987; Vaughn *et al.*, 1977). IPLB-LD-652Y stocks from two laboratories (Invasive Insect Biocontrol and Behavior Laboratory, USDA Beltsville, MD and USDA Forest Service, Delaware, OH) were used (see Supplementary Methods).

L. dispar eggs of the New Jersey Standard Strain were obtained from the USDA APHIS rearing facility, Otis Air National Guard Base, MA. Larvae were hatched and reared to the desired developmental stages on gypsy-moth artificial diet from Southland Products supplemented with 0.07 g l⁻¹ ferric citrate (Sigma no. F3388) at 28 °C in a 16 h : 8 h light : dark cycle.

Transfection of IPLB-LD-652Y cells. Cells were seeded in 6- or 12-well plates (1 or 0.5 × 10⁶ cells per well, respectively). Transfections were conducted in serum-free medium using Cellfectin Transfection Reagent (Life Technologies) following the supplier's protocol. Treatment reagents were exchanged for complete medium after 4 h of incubation at room temperature or at 28 °C. Control cells were not transfected and incubated in serum-free medium. For 12-well plates, 1.5–2 µg of mCherry or CrPV-1A transcript (see Supplementary Methods) or 1.5 µg of eGFP plasmid [pAcP(+)IE1eGFP (Harrison *et al.*, 2010; Jarvis *et al.*, 1996)] was used per well. The number of cells and amount of transfected nucleic acids were doubled when six-well plates were used. Samples of transfected cells and supernatants were collected at the indicated times post-transfection in microcentrifuge tubes and kept at –20 °C until RNA was extracted. The time-zero sample was collected immediately after replacement of treatment reagents with fresh medium. Samples for TEM were collected in the same way.

Inoculation of IPLB-LD-652, IPLB-LdFB and IPLB-SF21 cells.

Cells seeded in 12-well plates (0.3 × 10⁶ cells in 500 µl per well) were inoculated 1 or 2 days after seeding. For the mock treatment, the corresponding amount of medium was added instead of medium with virus. The LdIV1 inoculum was prepared from untreated IPLB-LD-652Y confluent cultures (more than 7 days after seeding, when

obvious CPEs were observed). Briefly, the contents of the flasks were recovered and cells were disrupted by passing through a 26G needle or by three cycles of freezing in liquid N₂ and thawing at 50 °C. Debris was removed by centrifugation at 3500 × g at 4 °C for 10 min and the supernatant was cleared through a 2 µm filter. Samples of recovered material (200 µl) were used to inoculate each well. Samples of cells and supernatants were collected at the indicated times and kept at -20 °C until RNA was extracted. The time-zero sample was collected immediately after inoculation.

Isolation of viral particles. Viral particles for TEM were released from cells into the medium of each sample (1.5 ml) by three freeze-thaw cycles, and cellular debris was removed by centrifugation as described above. Supernatant was passed through a 0.22 µm filter, and the filtrate was layered onto a 20% sucrose/0.01 M sodium phosphate (pH 7) cushion and then centrifuged at 124 000 × g at 4 °C for 4 h (Sorvall M150 micro ultracentrifuge). Pelleted particles were resuspended in 0.005 M sodium phosphate buffer (pH 7) and the cushion centrifugation was repeated. The final particle pellet was resuspended in 14 µl of 0.005 M sodium phosphate buffer (pH 7) and analysed by TEM. Viral particles for genome cloning and structural protein analysis were isolated from untreated cells and concentrated with PEG (Killington *et al.*, 1996) (see Supplementary Methods).

Microscopy. For TEM, 3 µl of particles was transferred to a carbon grid and stained with 3 µl of 2% uranyl acetate for 3 min. TEM was carried out with a JEM 2100 transmission electron microscope (JEOL) at the Microscopy and NanoImaging Facility of Iowa State University. Cell cultures transfected with the indicated treatments were examined daily under an optical inverted microscope to follow the development of CPEs. Samples from cells transfected with mCherry transcript or eGFP plasmid were analysed 1–3 days post-transfection to detect fluorescence and test transfection efficiency. Detection of fluorescence and final cell imaging was conducted using an AxiovertA.1 microscope (Carl Zeiss).

Viral genome cloning and sequencing. For the initial viral genome amplification, particles that had been extracted from cells analysed by TEM were used as template to generate products with degenerate primers (Table S1). The sequences of initial clones were used as reference to fill gaps by primer walking using as template viral RNA extracted from particles obtained from untreated cells. The viral genome was completed by 5' and 3' RACE (see Supplementary Methods).

Viral genome sequence. Initial sequence analysis was carried out using NCBI databases and software (Altschul *et al.*, 1997, 2005). Polyprotein cleavage sites were predicted using NetPicoRNA software (Blom *et al.*, 1996) or by sequence comparison with other iflaviruses. Polyprotein sequence identity and similarity (in %) to other iflaviruses were determined by EMBOSS Stretcher (www.ebi.ac.uk/Tools/psa/emboss_stretcher). Phylogenetic analysis was performed with MEGA5 software using the neighbour-joining method, a bootstrap test of 1000 replicates and a Poisson correction method (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.*, 2011; Zuckerkandl & Pauling, 1965).

Viral structural proteins. Viral particles enriched by PEG were analysed by SDS-PAGE using Novex precast gels (4–12% or 10%) and the NuPAGE system following the supplier's protocols (Life Technologies). Gels were transferred to PVDF membranes and the N termini of selected bands were sequenced by Edman degradation at the Protein Facility of Iowa State University (Table S3). Protein molecular mass was calculated using the online tool from Science Gateway (<http://www.sciencegateway.org/tools/proteinmw.htm>).

End-point RT-PCR. Total RNAs (0.2–1.3 µg) or viral RNAs from semi-pure particles (20 ng) (see Supplementary Methods) were used as templates to synthesize first-strand cDNAs with reverse primers for LdIV1 and actin (Table S1), using SuperScript III Reverse Transcriptase (Life Technologies). The same reverse primers were combined with their corresponding forward primers (Table S1) to amplify two fragments of the LdIV1 genome (1.2 kb and 0.4 kb) or actin (0.6 kb). Platinum Taq DNA polymerase PCR kit (Life Technologies) or GoTaq DNA Polymerase (Promega) was used with the following program: 95 °C for 2 min and 30 s, followed by 35 cycles of 94 °C for 40 s, 55.5 °C for 40 s and 72 °C for 45 s and a final hold of 72 °C for 3 min. Final products were visualized by electrophoresis in agarose gels and sequenced to confirm their identity.

RT-qPCR. We used the iTaq Universal SYBR Green One-Step kit (Bio-Rad) with 100 ng of total RNA (see Supplementary Methods) per sample to amplify a segment of the LdIV1 genome, and beta-actin and ATP-synthase gene transcripts by one-step RT-qPCR (Table S1). Amplifications were performed in a CFX384 thermocycler (Bio-Rad) following a program including a final melting curve to verify the specificity of the products: 50 °C for 25 min; 95 °C for 5 min; 40 cycles of 95 °C for 5 s and 58 °C for 30 s; and one cycle of 95 °C for 30 s, 55 °C for 30 s, followed by stepwise 0.5 °C increases (10 s at each step) from 55 °C to 95 °C. Quantification and statistical analyses were carried out using qBase+ software (Biogazelle) considering target- and run-specific amplification efficiencies, taking the initial time point of each treatment as calibrator (equal to 1). For experiments with the IPLB-LD-652Y cell line, normalization was conducted by using amplification data from two reference gene targets (beta-actin and ATP-synthase genes). For other cell lines (IPLB-LdFB, IPLB-Ld652 and IPLB-SF21) this strategy was not possible since ATP-synthase expression was induced by infection and beta-actin expression was repressed over time, failing the reference target-stability test performed in qBase+ (data not shown). In these cell lines, we measured relative quantities of LdIV1 in 100 ng of total RNA comparing every time point to the initial amounts of inoculum (taking time point zero as reference equaling 1).

ACKNOWLEDGEMENTS

The authors thank James Slavicek and Nancy Hayes-Plazolles (USDA Forest Service, OH) for supplying the IPLB-LD-652Y cell line and for advice on the maintenance of this cell line; Craig Mello and Don Gammon (RNA Therapeutics Institute, MA) for sharing the cell line IPLB-LD-652; Peter Christian (National Institute for Biological Standards and Control, UK) for providing CrPV; members of the Miller, Bonning and Toth laboratories for valuable discussions and comments on the manuscript; and anonymous reviewers for critical suggestions. This work was supported by the Iowa State University Plant Sciences Institute Virus-Insect Interactions Initiative, and USDA-NRI/AFRI Pest and Beneficial Insects in Plant Systems (award 2012-67013-19295).

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