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Biology of a new virus isolated from *Lupinus nootkatensis* plants in Alaska

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A new virus named Nootka lupine vein-clearing virus (NLVCV) was isolated from *Lupinus nootkatensis* plants that were confined to a relatively small area in the Talkeetna mountains of south-central Alaska. Annual surveys (2000–03) consistently found leaf symptoms of pronounced vein clearing and mosaic on 3- to 4-week-old plants in late June. Spherical particles ≈30 nm in diameter were isolated from these leaves. Virions contained a single-stranded RNA of ≈4·0–4·2 kb and one species of capsid protein estimated to be ≈40 kDa. The double-stranded RNA profile from naturally infected leaves consisted of three major bands ≈4·2, 1·9 and 1·5 kbp. Protein extractions from either sap or virions of diseased plants reacted to polyclonal antiserum made against the virions in Western blot assays. A predicted PCR product ≈500 bp was synthesized from virion RNA using primers specific to the carmovirus RNA-dependent RNA polymerase (RDRP) gene. The nucleotide sequence of the amplified DNA did not match any known virus, but contained short regions of identity to several carmoviruses. Only species belonging to the Fabaceae were susceptible to NLVCV by mechanical inoculation. Based on dsRNA profile, size of virion RNA genome and capsid protein, and similarity of the RDRP gene to that of other carmoviruses, it is suggested that NLVCV is a member of the family *Tombusviridae*, and tentatively of the genus *Carmovirus*. As the host range, RDRP gene and dsRNA profile of NLVCV are different from those of known viruses, this is a newly described plant virus.

Keywords: *Carmovirus*, Fabaceae, *Lupinus nootkatensis*, NLVCV, Nootka lupine vein-clearing virus, *Tombusviridae*

Introduction

Very little is known about the identity, quantity and distribution of plant viruses in natural plant ecosystems (Nienhaus and Costello, 1989; Stobbs *et al*., 1992; Latham & Jones, 1997; Costello *et al*., 2000; Hull, 2002). Intensive plant virus surveys are usually directed at economically important crops grown in artificial environments, and epidemiological studies involve virus movement within monocultures which may overlap with roadside weeds and adjacent cultivated land. Virus movement between natural ecosystems, crops and urban plants is largely ignored. There is very little knowledge of plant viruses in Alaska. Vast forests and wetlands in Alaska are interspersed with a few small agricultural regions, small, isolated villages, and several larger urban developments. In 1999 surveys of plant viruses in native plants were initiated on sites in close proximity to, but never used for, agricultural crops in the Matanuska Valley of south-central Alaska. In mid-August, in the Hatcher Pass Recreational Area along the Little Susitna River, several *Lupinus nootkatensis* plants were observed with prominent vein clearing on their leaves. It was hypothesized that the foliar discolorations were induced by a viral infection. This paper reports the characterization, identity, biology and epidemiology of a novel virus, referred to as Nootka lupine vein-clearing virus (NLVCV), isolated from *L. nootkatensis* plants in Alaska.

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Materials and methods

Virus source and collection of lupine tissue and seed

Lupinus nootkatensis plants with virus-like symptoms were observed along a 13 km trail bordering the Little Susitna River in the Hatcher Pass State Recreation Area in south-central Alaska in June–October 2000–03, and plants with and without these obvious symptoms were sampled. Leaves were detached from individual plants, recorded and stored at 4°C for use within 2 weeks, or at –80°C for long-term storage. Seed was collected from these plants in late summer and stored at room temperature. Lupine (≡ lupin) plants growing on other sites extending from the northern part of Alaska to Prince William

Sound were also sampled: Dalton Highway, Happy Valley (2000); Richardson Highway (2000–01); Paxton (2000); Palmer (2000–01); Portage Bay (2001); Cordova (2001); Coghill Lake (Prince William Sound, 2001); Kodiak (2003); Kuskokwim River (2003).

Virion purification

Virus was isolated and partially purified from field-collected *L. nootkatensis* leaves with distinct vein-clearing and mottling symptoms. Leaves from symptomless plants from the same site or other sites were similarly processed for comparison. A minipurification protocol for the isolation and partial purification of virions was used, based on those of Lane (1986, 1992). Leaf tissue (1 g) was homogenized with 20 mL extraction buffer (0·4 M sodium citrate, pH 6·5–7·0) and 0·15 mL 0·5 M sodium diethyldithiocarbamate in a Waring blender. The sap was expressed through wet muslin and centrifuged for 12 min at 111 000 ***g*** in a Beckman 50·2 Ti rotor (Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was filtered through Miracloth (CalBiochem, La Jolla, CA, USA), 0·3 mL 10% Triton X-100 was added and the mixture was inverted six to eight times, followed by a further centrifugation at 111 000 ***g*** for 45 min. Pellets were suspended in ≈150 μL sterile water and stored at –80°C. Larger sample weights were processed by increasing the proportions accordingly and combining the resulting pellets.

Electron microscopy

Leaf sap or partially purified particles derived from infected leaves were added to formvar-carbon-coated grids and stained with 2% uranyl acetate. Ultrathin sections of diseased and healthy leaf tissue were processed using standard procedures (Meek, 1976). Tissue was fixed in 3% glutaraldehyde and 2% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Spurr's epoxy resin (Spurr, 1969). Sections were double-stained with uranyl acetate and lead citrate (Reynolds, 1963). A Zeiss 10 CA transmission electron microscope was used at 60 kV.

Protein analysis

Virions were mixed with an equal volume of reducing buffer (62·5 mM Tris–HCl, pH 6·8, 2% SDS, 0·5% α-mercaptoethanol, 25% glycerol, 0·01% bromophenol blue) and boiled for several minutes, then 5–10 μL was applied to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (Laemmli, 1970). Electrophoresis was carried out using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The gels were stained with Coomassie Blue R-250 or used in Western analysis. Total leaf protein extractions were performed by grinding 0·1 g tissue in 1 mL dH_2O and processed as described for protein extraction from virions.

Virion RNA and double-stranded RNA extraction and analysis

Virion RNA was obtained by adjusting the virion concentration to 2 mg mL^{-1} in STE (0·1 M NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7·5), 0·5% SDS, 100 μg mL^{-1} proteinase K) and incubating for 60 min at 37°C. Following phenol/chloroform extraction and ethanol/sodium acetate precipitation (Maniatis *et al*., 1982), the RNA was dissolved in dH_2O and stored at –80°C. Extracted RNA and size markers were denatured and glyoxylated, and their size evaluated on a denaturing 1% glyoxal agarose gel using a Northern Max-Gly Kit (Ambion, Inc., Austin, TX, USA).

Double-stranded RNA (dsRNA) was extracted from naturally NLVCV-infected *L. nootkatensis* leaves (Valverde *et al*., 1990). Based on a procedure that separated ssRNA and dsRNA fractions (Chomczynski & Sacchi, 1987; T.J. Morris, University of Nebraska, Lincoln, NE, USA, unpublished results), 0·3 g leaf tissue from infected or healthy *L. nootkatensis* was ground in a mortar and pestle with 0·6 mL phenol, 0·6 mL extraction buffer (0·1 M Tris, 0·2 mM NaEDTA, 0·2 M NaCl pH 8·0, 1% SDS) and 25 μL α-mercaptoethanol, mixed with 0·6 mL chloroform, dispensed into microfuge tubes, vortexed and microfuged at 4°C for 5 min. The aqueous phase was transferred to a fresh tube and extracted with phenol/chloroform, followed by sodium acetate/ethanol precipitation. The dried pellet was suspended in 20 μL dH_2O, 100 μL 7 M lithium chloride added, and the mixture incubated on ice at 4°C overnight before centrifugation in a microfuge. The supernatant was transferred to a fresh tube, ethanol-precipitated, and the pellet suspended in ≈50 μL water. The dsRNA was treated with DNase 1 (Boehringer Mannheim, Inc., Indianapolis, IN, USA) as directed by the manufacturer. A tombusvirus, *Tomato bushy stunt virus* (TBSV) and a carmovirus, *Turnip crinkle virus* (TCV) were similarly processed from infected *Nicotiana benthamiana* plants (infectious clones provided by T.J. Morris) as dsRNA control standards. The dsRNA profiles were resolved by electrophoresis through 1% agarose gels in 1 × TBE containing ethidium bromide.

RT–PCR cloning, sequencing and comparative analysis of the NLVCV RDRP

A segment of the RNA-dependent RNA polymerase (RDRP) gene of NLVCV was cloned following the method of Morozov *et al*. (1995). Randomly primed cDNA was produced using AMV-RT (Promega, Madison, WI, USA) according to the manufacturer's directions. Two to 5 μL cDNA were mixed with 40 pmol each of the degenerate primers carmo-II and carmo-VI (Morozov *et al*., 1995), 2·5 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 0·2 mM of each dNTP in the provided 1× reaction buffer to a final volume of 100 μL. The PCR regime was 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 50°C for 3 min and 72°C for 5 min, with a final 72°C 10 min extension step. The PCR

products were detected by electrophoresis on 1% agarose gels in 1 × TBE containing ethidium bromide.

The ≈500 nt PCR products were cloned using the PCR primer *Pst*I and *Sal*I endonuclease restriction sites into pGEM3Z (Promega) and transformed into *Escherichia coli* XL-2 Blue (Stratagene, La Jolla, CA, USA). Insert sequences from both strands of the four selected recombinant plasmids were determined. The RDRP amino acid sequences deduced from clones pNLVCV-2 (GenBank accession no. AY584590) and pNLVCV-27, -35 and -39 were compared with those of some members of the family *Tombusviridae* from the GenBank database using the neighbour-joining tree generated by CLUSTALX with a bootstrap analysis of 1000 replicates (Thompson *et al*., 1997).

Antisera production and serological analysis of NLVCV

Two rabbits were given four subcutaneous injections of partially purified NLVCV particles (a total of 1·2 mg per rabbit) ≈2 weeks apart by Strategic Biosolutions, Newark, DE, USA. The first bleed was 2 weeks following the second injection, followed by two more bleeds 11 and 14 days after the third and fourth injections, respectively.

Western analysis was performed by transferring the proteins previously separated by 12% SDS–PAGE to nitrocellulose with a Trans-Blot SD semidry transfer cell (Bio-Rad) and detecting the proteins as directed with the Immuno-Blot Colorimetric assay kit (Bio-Rad) using goat antirabbit IgG-conjugated alkaline phosphatase at 1 : 3000 and NLVCV antiserum at 1 : 2000 dilutions.

Naturally NLVCV-infected *L. nootkatensis* tissue and partially purified virions extracted from the same plant were assayed for serological relatedness to distinct viruses of two genera in the family *Tombusviridae*. Agdia Pathoscreen Kits utilizing DAS–ELISA with alkaline phosphatase enzyme conjugate were used as directed for the carmoviruses *Carnation mottle virus* (CarMV), *Hibiscus chlorotic ringspot virus* (HCRSV), *Melon necrotic spot virus* (MNSV) and *Pelargonium flower break virus* (PFBV); and for the tombusvirus TBSV. Assays were followed according to the manufacturer's instructions with the provided antibody-coated microtitre plates, buffers, enzymes and positive controls. Frozen tissues (0·2 g) from nine different infected *L. nootkatensis* plants collected in 2002 were ground in 1 mL dH_2O, and 10 μL of the extract was diluted with 90 μL extraction buffer before applying to each antibody-coated well. Additional 100 μL aliquots of virions that were minipurified from the same plants and diluted (1 : 10) in extraction buffer were assayed on the same plate. The assays were analysed on a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm with positive readings at least three times that of the buffer control.

Lupinus nootkatensis seed germination

Seed was collected from *L. nootkatensis* plants from Hatcher Pass in which no symptoms or associated viral protein and particles could be detected. They were treated with 95–98% sulphuric acid for 30 min, then washed in distilled water several times before planting into vermiculite in small trays. Seedlings were germinated in a growth chamber at 20°C, transplanted into containers and transferred to a glasshouse with natural light and temperatures that ranged between 10 and 30°C from June to September. *Lupinus nootkatensis* seed collected from naturally NLVCV-infected plants from the same site, and presumed healthy seed from the Kenai Peninsula (Alaskan Seeds, Inc., Kenai, AK, USA), were similarly processed.

Transmission tests to determine the host range of NLVCV

Leaf tissue with symptoms was ground with 0·01 M phosphate buffer (1 g per 10 mL) and a dash of Celite 545 in a mortar and pestle, and applied with a cotton swab to the leaves of selected plant test species. Partially purified NLVCV preparations were similarly used for inoculations.

Lupinus nootkatensis seedlings derived from apparently healthy plants were inoculated with sap from plants with symptoms before being transferred to a glasshouse with natural light and a 10–30°C temperature range. Other lupine species, as well as other leguminous species and various typical virus-indicator species, were similarly inoculated at the seedling stage and observed in growth chambers at 20°C with 14 h fluorescent lights.

All plants were assayed 2–3 weeks after inoculation by Western blot analysis of leaf protein extracts from both the inoculated leaf and new leaves. Additional virus minipurifications and Western analyses were performed from selected plants between 4 and 15 weeks after inoculation.

Results

Virus incidence and symptom expression

The first obvious symptoms in naturally infected *L. nootkatensis* plants were distinct vein clearing and/or mosaic patterns on leaves of mature plants (Fig. 1). Vein clearing usually appeared on the oldest leaves first, and over time became apparent on the younger leaves on the upper portion of the plant. The number of naturally NLVCV-infected plants in field samples in 2000, 2001, 2002 and 2003 were 31 out of 81 (38%); 23 out of 46 (50%); 22 out of 44 (50%); and 45 out of 75 (60%), respectively. All NLVCV-infected plants were confined to the Hatcher Pass region adjacent to the Little Susitna River (2000–03) from the headwaters to ≈4·5 km downstream, while several kilometres north-east along the Archangel River (2000), two plants out of 15 were infected. NLVCV capsid protein was always detected by Western analysis in mature plants with leaf symptoms, but only rarely in samples from symptomless plants. As plants matured virus concentration, as determined by virion extraction and Western analysis, appeared to increase in the leaf tissue until late senescence.

Figure 1 Typical vein-clearing symptoms on leaf of *Lupinus nootkatensis* naturally infected with NLVCV.

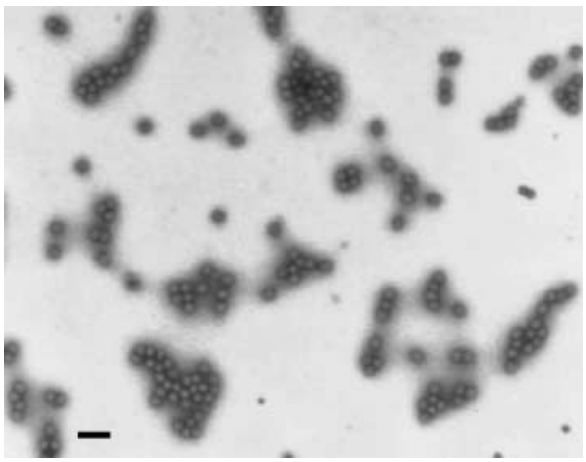

Figure 2 Electron micrograph of partially purified NLVCV stained with uranyl acetate. Bar = 100 ηm.

Seed transmission of NLVCV

No plants that germinated from *L. nootkatensis* seeds collected from infected, symptomless plants or those obtained commercially from the Kenai Peninsula (102, 50 and 35 seeds, respectively) displayed symptoms or tested positive for NLVCV by Western analysis from total leaf protein extractions.

Plant host range of NLVCV

Transmission of NLVCV from sap of naturally infected *L. nootkatensis* occurred in 90% of inoculated *L. nootkatensis* seedlings (27 out of 30). Other lupine species susceptible to sap inoculation included *Lupinus albus*, *Lupinus augustifolia*, *Lupinus luteus*, *Lupinus nana*, *Lupinus succulentus*, *Lupinus texensis* cvs Bluebonnet and Maroon, and *Lupinus versicolor*. Systemic symptoms on young *L. nootkatensis* plants included mild mottling and occasional vein clearing, while the other lupine species expressed severe mottling, vein clearing, leaf necrosis and death. Virions purified from experimentally infected *L. nootkatensis* plants and inoculated into *L. nootkatensis*, *L. succulentus* and *L. texensis* cvs Bluebonnet and Maroon resulted in symptoms similar to those described for the original sap-inoculated plants. NLVCV capsid protein was detected in all susceptible plants from total protein extractions and/or virus minipurifications, and never from symptomless inoculated plants.

Chickpea (*Cicer arietinum*) showed a pronounced and immediate response to NLVCV when seedlings were inoculated with sap (13 out of 15 plants) or virions (11 out of 12 plants) from infected *L. nootkatensis* plants. Leaves developed mottling and some vein clearing, and turned yellow or necrotic before the branchlets wilted or the main stem collapsed, resulting in plant death within 7 days after inoculation with virions, or within 2 weeks after sap inoculation. Sap from NLVCV-infected *C. arietinum* plants was infectious, causing disease in 12 out of 13 healthy *C. arietinum* plants. Furthermore, the disease was transmissible back to lupine species (as judged by symptoms and Western analysis of total protein leaf extractions), by sap or partially purified virions derived from infected *C. arietinum*, as follows (infected number/total number): *L. texensis* cv. Bluebonnet (3/4 sap, 4/4 virion); *L. texensis* cv. Maroon (4/4 sap, 4/4 virion); and the natural host, *L. nootkatensis* (1/8 sap, 1/8 virions).

Sap or virions from naturally NLVCV-infected *L. nootkatensis* were transmitted mechanically to *Vigna unguiculata* leaves, but infection was restricted to the inoculated leaf. Similar inoculations did not transmit NLVCV to several other members of the Fabaceae, including *Melilotus officinalis*, *Medicago media*, *Pisum sativum* and *Trifolium pratense*, or to some of the commonly used experimental plant hosts including *Chenopodium quinoa*, *Chenopodium amaranticolor*, *Gomphrena globosa*, *Nicotiana benthamiana*, *Nicotiana clevlandii*, *Nicotiana debneyi* and *Nicotiana tabacum* cvs Samsun and White Burley.

Virus purification and electron microscopy

Partially purified virions, isolated and concentrated by differential centrifugation from naturally infected lupine plants collected in September 1999, from mature plants yielded ≈13 mg per 100 g tissue (assuming the same 4·5 extinction coefficient as TBSV), and had an absorbance ratio (A_{260}/A_{280}) of 1·46, uncorrected for light scattering. The virus concentration was usually considerably lower from young plants collected in early June from the 2001–02 samples, as judged by Western analysis. The particles stained readily with uranyl acetate and appeared as ≈30 nm isometric particles (Fig. 2). Ultrathin sections of leaf material displayed aggregates of virus particles and scattered vacuoles in the mesophyll (Fig. 3).

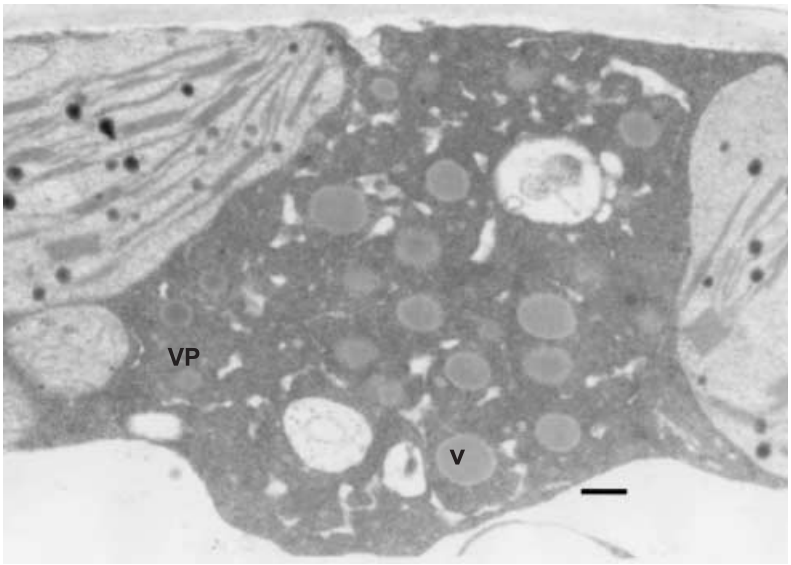

Figure 3 Ultrathin section of *Lupinus nootkatensis* mesophyll cell depicting virus particles (VP) of Nootka lupine vein-clearing virus and vacuoles (V). Bar = 250 ηm.

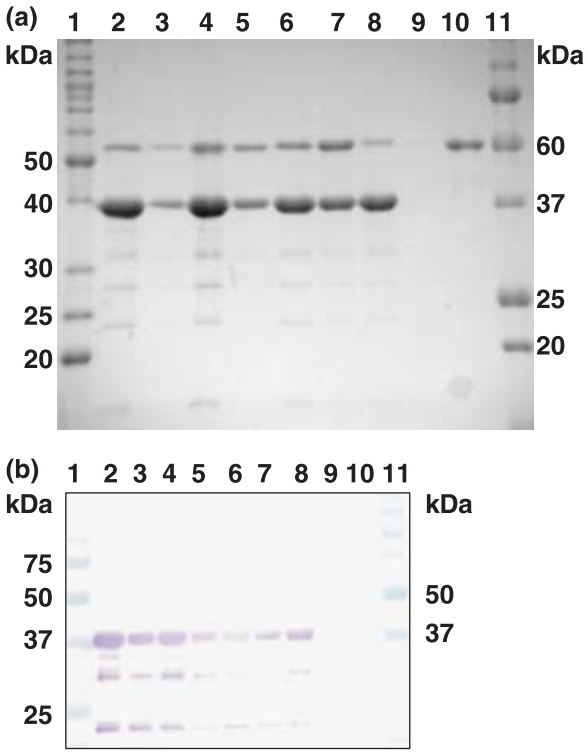

Figure 4 Protein analysis of NLVCV. Proteins extracted from partially purified virions, fractionated in a 12% SDS–PAGE gel and (a) stained with Coomassie Blue (lanes: 1, Invitrogen BenchMark protein ladder; 2–8, seven plants with symptoms; 9 and 10, two symptomless plants; 11, Bio-Rad prestained Precision Plus Protein Standards, All Blue), or (b) blotted onto nitrocellulose and used in Western analysis with NLVCV antiserum (lanes: 1 and 11, Bio-Rad prestained Precision Plus Protein Standards, All Blue and Dual Color, respectively; 2–8, seven different plants with symptoms; 9 and 10, two different symptomless plants).

Virion protein analysis and ELISA

A major protein of ≈40 kDa, presumed to be the coat protein, extracted from virions (Fig. 4a), was specifically recognized on Western blots (Fig. 4b) using the polyclonal antiserum generated against NLVCV virions.

NLVCV-infected tissue or purified virions did not react with CarMV, HCRSV, MNSV, PFBV or TBSV antisera by ELISA.

Analysis of virion nucleic acid and dsRNA

Virion nucleic acid resolved on a denaturing 1% agarose gel as a single-stranded RNA species ≈4·2 kb (Fig. 5a). Three

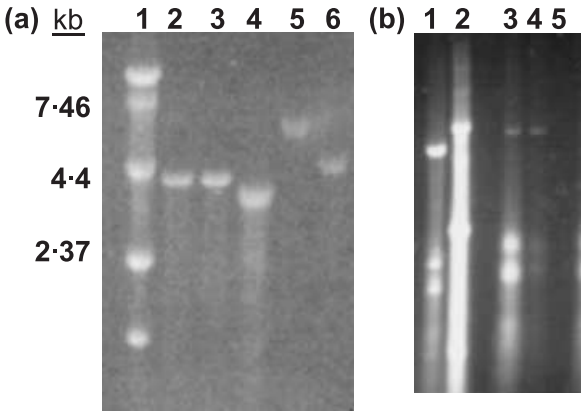

Figure 5 Analysis of NLVCV RNA. (a) Genomic NLVCV RNA extracted from partially purified virions and separated on a denaturing 1% glyoxal agarose gel. Lanes: 1, RNA molecular weight marker; 2 and 3, two different NLVCV preparations; 4, TCV RNA (4·05 kb); 5, *Foxtail mosaic virus* RNA (6·15 kb); 6, TBSV RNA (4·77 kb). (b) Double-stranded RNA species extracted from virus-infected plant leaves and separated on 1% agarose in 1 × TBE. Lanes: 1, TCV-infected *Nicotiana benthamiana*; 2, TBSV-infected *N. benthamiana*; 3 and 4, two NLVCV-infected *Lupinus nootkatensis* plants; 5, symptomless *L. nootkatensis*.

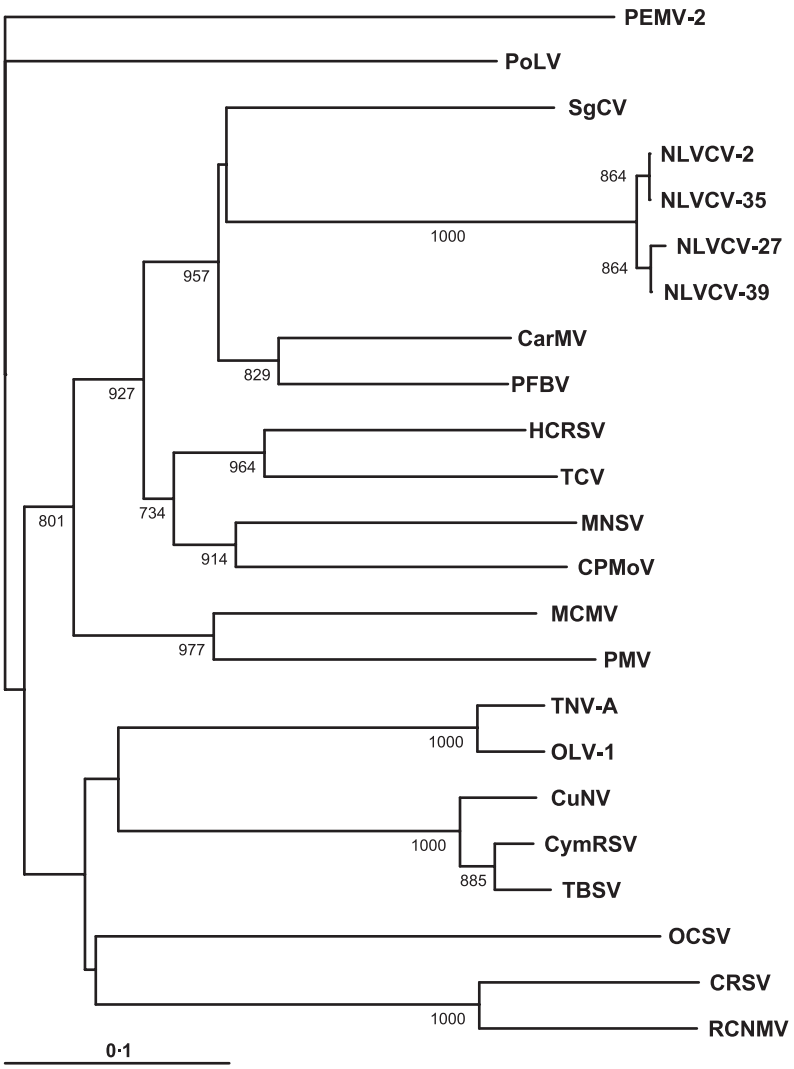

Figure 6 Phylogenetic tree showing relationships of four NLVCV clones and selected members of the family *Tombusviridae* based on alignments of conserved RDRP amino acid sequences. Viruses included [genus: species (acronym, accession number)] – *Aureusvirus*: *Pothos latent virus* (PoLV, X87115); *Avenavirus*: *Oat chlorotic stunt virus* (OCSV, X83964); *Carmovirus*: *Carnation mottle virus* (CarMV, X02986), *Cowpea mottle virus* (CPMoV, U20976), *Hibiscus chlorotic ringspot virus* (HCRSV, X86448), *Melon necrotic spot virus* (MNSV, M29671), *Pelargonium flower break virus* (PFBV, AJ514833), *Saguaro cactus virus* (SgCV, U72332), *Turnip crinkle virus* (TCV, M22445); *Dianthovirus*: *Carnation ringspot virus* (CRSV, L18870), *Red clover necrotic virus* (RCNMV, J04357); *Machlovirus*: *Maize chlorotic mottle virus* (MCMV, X14736); *Necrovirus*: *Olive latent virus-1* (OLV-1, X85989), *Tobacco necrosis virus A* (TNV-A, M33002); *Panicovirus*: *Panicum mosaic virus* (PMV, U55002); *Tombusvirus*: *Cucumber necrosis virus* (CuNV, M25270), *Cymbidium ringspot virus* (CymRSV, X15511), *Tomato bushy stunt virus* (TBSV, U80935). *Pea enation mosaic virus-2* (PEMV-2, U03563) is a member of the genus *Umbravirus* and was used as an outgroup.

distinct dsRNA species were found from NLVCV-, TCV- and TBSV-infected tissues (Fig. 5b). The TCV (4·0, 1·7 and 1·5 kbp) and TBSV (4·7, 2·1 and 0·9 kbp) profiles (Morris, 2001) served as size references for estimating the following dsRNA species for NLVCV: ≈4·2, ≈1·9 and ≈1·5 kbp.

PCR, cloning and sequencing

A conserved motif of the carmovirus-related RDRP gene was amplified from NLVCV genomic RNA with the predicted product size of ≈500 nt. The nucleotide sequences of four independent cDNA clones, pNLVCV-2, -27, -35 and -39, were nearly identical. Carmovirus members that had regions of amino acid homology (pairwise percentage identity) >60% were: CarMV (70%); HCRSV (61%); PFBV (66%); *Saguaro cactus virus* (SgCV, 66%). A phylogenetic tree (Fig. 6) that included representatives from all genera in the family *Tombusviridae* clearly shows NLVCV clustered within the carmoviruses.

Discussion

A new virus disease in *L. nootkatensis* plants from a naturally occurring population in Alaska was documented after extensive field observations and laboratory studies. Experimental plant-inoculation studies demonstrated that a new virus isolated from diseased field plants could be transmitted effectively to *L. nootkatensis* seedlings, and resulted in similar symptoms. The causal agent of the disease has been named as Nootka lupine vein-clearing virus (NLVCV).

Properties of NLVCV correspond to criteria defining viruses in the family *Tombusviridae* (van Regenmortel *et al*., 2000). These include an isometric particle of ≈30 nm with a capsid protein of ≈40 kDa that encloses a monopartite, single-stranded RNA genome of between 4·2 and 4·3 kb. Viruses in this family are subdivided into eight genera, with NLVCV matching most closely to the genus *Carmovirus*. The occurrence of numerous virus particles and scattered vacuoles in the cytoplasm of

mesophyll cells from NLVCV-infected lupine leaves is normal for carmoviruses. As is typical of tombusviruses and carmoviruses (Díez *et al*., 1998; Morris, 2001), partially purified virions of NLVCV were good immunogens, yielding antisera that specifically bound the viral coat protein. Presumed degradation of the capsid species often occurred, producing smaller fragments that were readily observed on gels stained with Coomassie Blue and confirmed as coat protein by Western analysis. Distinct viruses in the family *Tombusviridae* tend to be serologically distinct (Koenig & Gibbs, 1986; Díez *et al*., 1998; Morris, 2001). Purified NLVCV particles were determined to be serologically distinct from four carmoviruses (CarMV, HCRV, MNSV, PFBV) and one tombusvirus (TBSV), as it did not react to antisera to those viruses.

The dsRNA profile representing replicative dsRNA from NLVCV-infected leaves resembled those of carmoviruses (Brunt *et al*., 1996; Heaton, 2001) rather than tombusviruses (Brunt *et al*., 1996; Morris, 2001), with the largest dsRNA species being ≈4·2 kbp and possessing two smaller subgenomic dsRNA species of ≈1·9 and 1·5 kbp, similar to the range found in carmoviruses. Only genomic RNA is encapsidated by NLVCV, unlike some carmoviruses and tombusviruses which also encapsidate subgenomic, satellite and defective interfering RNAs (van Regenmortel *et al*., 2000).

Additional evidence that NLVCV shares similarity with other carmoviruses was obtained from RT–PCR using a carmovirus-specific primer assay in which a PCR product of the expected size of ≈500 nt was obtained from infected *L. nootkatensis* plants. Moreover, the sequence of this PCR product contained conserved regions of nucleotide and deduced amino acid sequences common to carmoviruses within the RDRP gene (Morozov *et al*., 1995), but did not share close homology with any virus currently in the GenBank database.

Carmoviruses are known to have narrow natural host ranges and are geographically limited. NLVCV is found only in an isolated region in the Talkeetna mountain range, and its natural host range is thus far limited to one plant species, *L. nootkatensis*. The natural mode of transmission is unknown for NLVCV, as is the case for the majority of viruses in the family *Tombusviridae*. A few members of this family have been found in rivers and lakes (Tomlinson *et al*., 1983; Koenig, 1986; 1988), irrigation water (Yi *et al*., 1992), or soil, and several are vectored by chytrid fungi (Hibi & Furuki, 1985; Osaki *et al*., 1988; Rochon & Tremaine, 1988; Campbell *et al*., 1995; Campbell, 1996). Preliminary soil transmission tests resulted in no NLVCV transmission when *L. nootkatensis* seedlings were germinated and grown in soil removed from the roots of naturally infected lupine plants (data not shown). Interestingly, infected *L. nootkatensis* plants were found only on or very close to the riverbank, where flooding may occur several times a year. Experimentally, NLVCV is very stable and relatively easy to transmit to lupine species by rubbing infected sap or purified particles onto seedlings. Spring snowmelt in the Talkeetna mountain range contributes to river and stream floods with extremely turbulent water that is especially abrasive to surrounding vegetation. Consequently, if NLVCV is waterborne in nature, the virus could easily be transmitted to *L. nootkatensis* seedlings by strong water action. Infected *L. nootkatensis* plants have been identified from the headwaters to ≈4·5 km downstream of the Little Susitna River. Thus the Mint Glacier, a major tributary of the headwaters, may be implicated as the site of NLVCV origin.

Although most carmoviruses and NLVCV have small natural host ranges, most carmoviruses have large and diverse experimental host ranges. NLVCV infects only some species in the Fabaceae. The limited experimental host range does not enable easy maintenance and propagation as the majority of the non-native lupine plants and *C. arietinum* died within 1 month following initial infection, while in *V. unguiculata* infection occurred only on the inoculated leaf and at low virion concentrations. Presently *C. arietinum* is the best indicator host for rapid NLVCV-infectivity assays. *Lupinus nootkatensis*, the natural host of NLVCV, exhibits tolerance to the virus, as inoculated seedlings do not exhibit the immediate stem wilting and plant collapse incurred by other susceptible species. Indigenous lupine plants with NLVCV have distinct vein clearing and some mosaic, with no other apparent abnormalities in habit, plus they produce viable seed. Virions of NLVCV were isolated from roots of infected *L. nootkatensis* and *C. arietinum* plants, but the concentrations were usually much higher in leaves (data not shown). During 4 years of field observations and laboratory assays, NLVCV was rarely found in young emerging plants, while the highest concentrations of NLVCV were found in leaves from infected older plants. As *L. nootkatensis* is perennial, the virus probably overwinters in the roots at relatively low levels and then moves systemically through the plant as it grows in the spring, reaching the highest concentrations as the plant attains maturity in the summer. Although the majority of carmoviruses are found in temperate regions, those that infect legumes are more commonly found in the tropics (van Regenmortel *et al*., 2000). The exceptions are *Pea stem necrosis virus* isolated from pea in Japan (Suzuki *et al*., 2002) and NLVCV. NLVCV is the first instance of a virus in the family *Tombusviridae* found naturally infecting a lupine species. Based on virion size and shape, sizes of coat protein and RNA genome, and transmissibility, it is proposed that NLVCV is a member of the family *Tombusviridae*. Furthermore, given the unique plant host range, dsRNA profile and similarity of RDRP sequence, it is suggested that NLVCV is a distinct new virus and a tentative member of the genus *Carmovirus*.

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