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BRIEF REPORT

Studies on biology and genomic characterization of a caulimo-like virus associated with a leaf distortion disease of *Lamium maculatum*

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Abstract A virus associated with leaf distortion of *Lamium maculatum* had features characteristic of caulimoviruses. The viral genome (Genbank accession number: EU554423) is 7,713 bp in size, with six open reading frames similar in size and organization to those of known caulimoviruses. Phylogenetic analyses based on the conserved ORF V polyprotein coding region indicated that the *Lamium* virus is a possible new member of the genus *Caulimovirus.* The virus was not transmitted by mechanical or graft inoculation, or by *Myzus persicae*. Because proof of pathogenicity remains to be demonstrated, the virus was named provisionally *Lamuim* leaf distortion-associated virus (LLDAV).

All plants of the perennial landscape ornamental *Lamium maculatum* "Beacon Silver" (*Lamiaceae*) obtained from commercial nurseries in three locations in the USA were affected by a growth disorder characterized by severe leaf deformation (Fig. 1a). Spherical virus-like particles 45–52 nm in diameter (Fig. 1b) were observed by transmission electron microscopy (TEM) in negatively stained partially purified leaf tissue extracts [1] from symptomatic plants but were not detected in similar preparations from asymptomatic plants. Preliminary tests revealed that the spherical virus-like particles associated with the *Lamium* leaf distortion disorder contained dsDNA similar in size and electrophoretic profile to gemonic DNA of caulimoviruses. Further studies, the results of which are reported below, were undertaken to establish the identity and properties of the *Lamium* virus and its possible relationship to members of the genus *Caulimovirus.* The only other virus known to occur in *Lamium* is *Lamium mild mosaic virus* (*Fabavirus*) [11].

Plants of *L. maculatum* "Beacon Silver" showing severe leaf distortion symptoms (Fig. 1) were obtained from commercial nurseries in Minnesota, Michigan and North Carolina. Asymptomatic plants of the same cultivar were obtained from a nursery in Norwich, UK. Symptomatic plants were used as virus source and asymptomatic plants as indicator plants throughout this study.

Virus detection assays were done by TEM using partially purified leaf tissue extracts. Disease and virus transmission tests were done using mechanical inoculation and aphid *Myzus persicae.* Indicator plants were either grown from seed or propagated clonally from source plants tested by TEM using partially purified extracts. Carborundum-dusted leaves of healthy indicator plants were rub-inoculated using crude sap extracts of infected "Beacon Silver" leaf tissue prepared in 100 mM potassium phosphate, pH 7.5, 0.5% (v/v) 2-mercaptoethanol. Graft inoculation was done by approach grafting between rooted healthy and infected "Beacon Silver" plants. Non-viruliferous apterae of *M. persicae* were allowed short (2–5 min) and long (24 h) acquisition access periods on infected "Beacon Silver" plants, transferred to healthy "Beacon Silver" for an inoculation access period of 12–24 h, and then eliminated by insecticide application. Indicator plants were maintained in the greenhouse at 22–26°C for up to 12 months post-inoculation, were observed for disease

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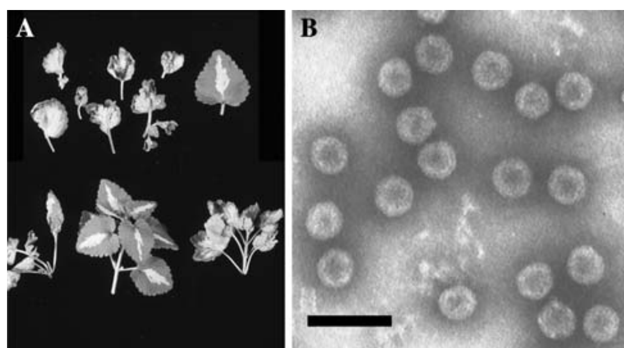

Fig. 1 **a** Symptoms associated with *Lamium* leaf distortion associated virus (LLDAV) in *Lamium maculatum* "Beacon Silver" (*top left*). Healthy leaves at *top right* and at *bottom middle*. **b** Virions of *Lamium* leaf distortion associated virus (LLDAV) negatively stained with ammonium molybdate. *Scale bar* represents 100 nm

symptoms, and were assayed for the presence of virus by TEM examination of negatively stained partially purified leaf tissue extracts or by Southern blot analysis. Five test plants per treatment were used in all transmission experiments. Serological tests were done by immunosorbent electron microscopy as described previously [13] using rabbit antisera to the following caulimoviruses: cauliflower mosaic virus (CaMV) (provided by T. Guilfoyle); dahlia mosaic virus (DaMV) and carnation etched ring virus (CERV) (provided by A.A. Brunt), and tobacco vein clearing virus (TVCV) [14]. These experiments were all done before the LLDAV genomic sequence was available to permit PCR-based virus indexing.

Purification of virions from infected "Beacon Silver" leaf tissue and extraction of genomic DNA from purified virions was done as described for TVCV [14]. Intact circular viral genomic DNA was linearized by digestion with SalI and cloned into the SalI site of pBluescript. Both strands of the cloned DNA were sequenced by primer walking (DNA Sequencing Facility, Iowa State University, Ames, IA). Sequence analyses were done using Geneworks (Intelligenetics Mountainview, CA) and Sequencher (Gene Codes Corporation, Ann Arbor, MI) programs. Database searches were conducted using FASTA and BLAST, including the Blastn, BlastP and tBlastn programs.

Sequence alignment was done by ClustalW (http://www.cbi.ac.uk/clustalw/index.html), and PHYLIP (http://evolution.genetics.washington.edu/phylip.html) software was used to construct a phylogenetic tree using the conserved ORF V region containing the aspartic protease (AP), reverse transcriptase (RT), and ribonuclease H (RH) domains.

Southern blot analyses were performed to determine whether LLDAV genomic sequences were present in the *Lamium* genome and also to test inoculated indicator plants for transmission. A CTAB method [7] was used to isolate and purify genomic DNA from noninfected *L. maculatum* "Beacon Silver" (UK), *L. maculatum* "Shell Pink" and infected *L. maculatum* "Beacon Silver" (MN) vacuum-dried plant tissues. Fifteen micrograms of total genomic DNA were digested with 20 units of *Eco*RI and *Hin*dIII. The digested DNA was separated in a 0.8% TBE agarose gel. After the DNA was depurinated by soaking the gel in 0.25 N HCl for 20 min, the DNA was cleaved and denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min. The gel was then neutralized by soaking in 1.0 M Tris–HCl (pH 8.0), 1.5 M NaCl for 30 min. The DNA on the gel was then transferred to a nylon membrane (MSI Magnacharge, Micron Separations, Westborough, MA) and was immobilized by UV cross-linking with a Bio-Rad GS gene linker (Bio-Rad laboratories, Hercules, CA) or by baking at 80°C in a vacuum oven for 2 h. The LLDAV clone was labeled with [α-^{32}P] dCTP using a Prime-a-gene labeling kit (Promega, Madison, WI) according to the manufacturer's instructions. Hybridization was done at 62°C overnight in a solution containing 5× SSC, 1× Denhardt's, 0.05 M Tris–HCl (pH 8.0), 0.01 M EDTA (pH 8.0), 1% SDS, and 200 μg/ml herring sperm DNA. Following hybridization, the hybridized membranes were washed under high-stringency conditions and were exposed to X-ray film for 3–7 days at −80°C, which was then developed. The highest-stringency wash was performed in 0.1× SSC, 0.5% SDS at 65°C.

The caulimo-like virus (Fig. 1b) associated with the leaf distortion disease occurring in *L. maculatum* "Beacon Silver" and subsequently named *Lamium* leaf distortion associated virus (LLDAV) was detected consistently by TEM in symptomatic plants from the three locations (Minnesota, Michigan, and North Carolina) in the USA but was not detected in asymptomatic "Beacon Silver" plants from the UK. Plants of "Beacon Silver" infected with the virus showed symptoms of severe leaf deformation, stunting and reduced vigor (Fig. 1a) under both greenhouse and outdoor landscape conditions during 3 years of observation. No disease symptoms were observed or virus detected in any of the following *L. maculatum* cultivars: "Chequers", "Orchid Frost", "Pink Pewter" and "White Nancy".

Detection of the *Lamium* virus by TEM using partially purified extracts was found to be satisfactory and reliable, but there was no experimental basis for assessing the relative sensitivity of this procedure compared to alternative assay techniques such as PCR amplification. The virus was not transmitted by mechanical inoculation to any of the following indicator plants: *Lamium maculatum* "Beacon Silver", "Chequers", "Orchid Frost", "Pink Pewter" and "White Nancy", *Nicotiana benthamiana*, *N. debneyi*, *N. occidentalis.* No local or systemic symptoms were observed in inoculated plants, and no virions were detected

by TEM in partially purified leaf tissue extracts or by Southern blot analysis. The virus was also not transmitted from infected to healthy "Beacon Silver" either by grafting or by *M. persicae* after either short or long acquisition access periods. In ISEM tests, the virions of the *Lamium* virus were neither trapped nor decorated with antibodies to CaMV, DaMV, CERV, or TVCV.

The 7,713-bp genome of LLDAV has the features that have been described for other caulimoviruses [9]. The protein encoded by each of the six ORFs has the highest identity and is of similar size to the corresponding ORF of CaMV as follows: ORF I (movement protein, 323 aa), 59% identity to CaMV NP_056724; ORF II (aphid transmission factor, 168 aa), 59% identity to CaMV_1104230A; ORF III (virion-associated protein, 122 aa), 44% identity to CaMV P03553; ORF IV (coat protein, 509 aa), 44% identity to CaMV NP_056727; ORF V (replicase, 696 aa), 67% identity to CaMV P03556; ORF VI (inclusion body/transactivation factor, 517 aa), 37% identity to CaMV P03557. A phylogenetic tree based on ORF V shows that LLDAV forms a clade with CaMV and HLV (Fig. 2).

When blots containing digested DNA from healthy *L. maculatum* "Beacon Silver" (UK) and infected *L. maculatum* "Beacon Silver" (USA) were probed with a full-length labeled LLDAV clone, hybridization was detected only to DNA from the infected plant (Fig. 3) The total size of the hybridized fragments for both *Eco*RI and *Hin*dIII digests was approximately 7.7 kb, which is the size of the full-length LLDAV genome, suggesting that the observed hybridization was to LLDAV genomic sequences liberated from virions during plant genomic DNA extraction.

The virion and genome properties of the LLDAV clearly identify it as a member of the genus *Caulimovirus.* Plants infected with LLDAV were also found (data not shown) to contain cytoplasmic inclusion bodies characteristic of caulimoviruses and encoded by ORFV. The sequence comparisons mentioned above and the phylogenetic analysis shown in Fig. 2 show that LLDAV is a member of a new and distinct species in the genus *Caulimovirus*, with only 37–67% amino acid sequence identity to known

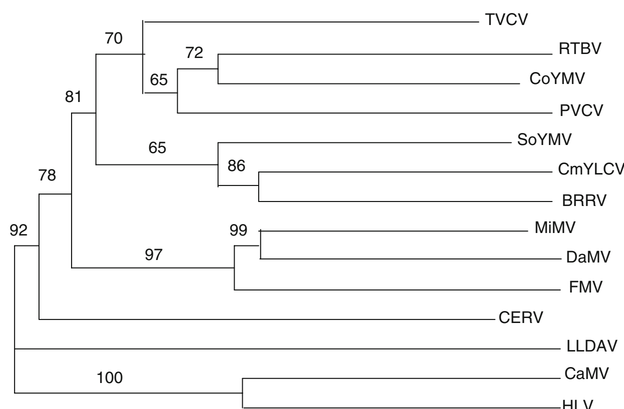

Fig. 2 Phylogenetic tree indicating the relationship of *Lamium* leaf distortion-associated virus (LLDAV) to other members of the family *Caulimoviridae* based on the conserved ORF V region containing the aspartic protease (AP), reverse transcriptase (RT), and ribonuclease H (RH) domains. Bootstrap probability values (%) for 500 replicates are shown in the branches. GenBank accession numbers for sequences used in the analysis are: cauliflower mosaic virus (CaMV, NC 001497), Cestrum yellow leaf curling virus (CmYLCV NC 004324), Commelina yellow mottle virus (CoYMV, NC 001343), carnation etched ring virus (CERV, NC 003498), horseradish latent virus (HLV, AY534732) and dahlia mosaic virus (DaMV, AY309479), figwort mosais virus (FMV, NC 003554), Mirabilis mosaic virus (MiMV, NC 004036), blueberry red ring spot virus (BBRV NC 003138), soybean chlorotic mottle virus (SoyMV, NC 001739), petunia vein clearing virus (PVCV, NC 001839), rice tungro bacilliform virus (RTBV, NC 001914), and tobacco vein clearing virus (TVCV, NC 003378)

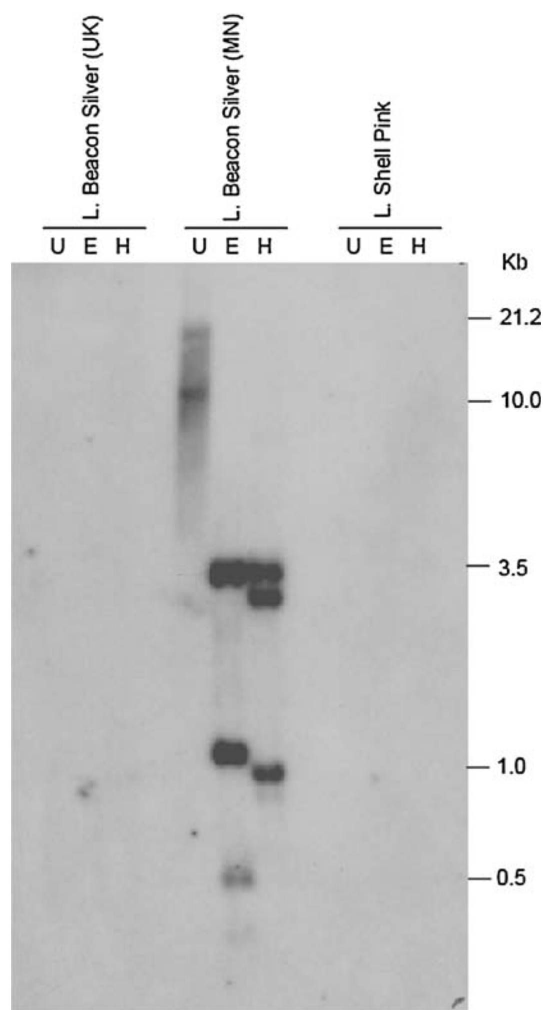

Fig. 3 Southern blot hybridization of *Lamium* genomic DNA, undigested (*U*), digested with *Eco*RI (*E*) and digested with *Hin*dIII (*H*). For Southern blot hybridization, DNA was transferred to a nylon membrane and probed with a labeled full-length LLDAV genomic clone. Plants and restriction enzymes are listed at the *top* of each *lane*, and positions of the molecular weight marker sizes are shown at the *right side* of the blot

caulimoviruses across the six putative protein-encoding regions of the viral genome.

The novel and interesting biological feature of LLDAV is its apparent absence of horizontal transmission by mechanical, aphid or graft inoculation. While many known caulimoviruses can be transmitted by mechanical inoculation to at least a restricted number of host plant species, there are several which have been transmitted experimentally only by aphid vectors (e.g. strawberry vein banding virus, SVBV) [5] or grafting (e.g. blueberry red ringspot virus) [8]. The aphid vector *Myzus persicae* failed to transmit LLDAV from infected to healthy *L. maculatum* "Beacon Silver" following both brief and extended acquisition feeding. This result should, however, be interpreted with caution. The *M. persicae* biotype used in this study readily colonized *L. maculatum*, and therefore while host preference or feeding behavior cannot be invoked as probable causes of virus transmission failure, it is possible that LLDAV may be transmitted by aphid vectors other than *M. persicae*. Aphid vector specificity has been reported, for example, for SVBV [6]. An attempt to transmit LLDAV by grafting was also unsuccessful. While this result must again be interpreted with caution because of the limited scope of the experiment as described above, it should be pointed out that another novel caulimovirus, TVCV, was also not transmitted by graft-inoculation and that plant cryptic viruses (*Partitiviridae*) are also reported to be not transmissible by graft-inoculation [2].

The similarities between LLDAV and TVCV in both genomic and biological properties beg the question of whether LLDAV may be transmitted only vertically via activatable viral sequences integrated in the host genome [14]. The results of Southern hybridization reported above indicate that LLDAV sequences are not integrated into the host genome and that the TVCV model of uniquely vertical virus transmission is not applicable to LLDAV.

All plants of *L. maculatum* "Beacon Silver" originated from a single seedling selection in the UK in 1976 [15], and neither LLDAV nor leaf distortion symptoms were detected in plants of the cultivar in the UK. This implies that LLDAV was at some point transmitted into the source of the infected "Beacon Silver" now grown in the US and that the virus has subsequently lost the ability to be transmitted vertically and is therefore now only transmitted horizontally by clonal propagation.

Loss of aphid transmissibility following repeated mechanical transmission or prolonged vegetative propagation has been documented for several plant viruses [3, 4, 10]. While there are no similar published reports of loss of transmissibility by mechanical inoculation, we have recently observed that a number of viruses (Arabis mosaic virus, cucumber mosaic virus, alfalfa mosaic virus, dasheen mosaic virus) ([4] and unpublished) that are known to be readily transmitted by mechanical inoculation are not transmitted in this manner from infected vegetatively propagated perennial ornamentals. It is possible that these viruses, like LLDAV, have for some undetermined reason lost the ability to be transmitted by mechanical inoculation.

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