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Nigerian tobacco latent virus: a new *Tobamovirus* from tobacco in Nigeria

J.L. Ladipo[1], R. Koenig[2,*] and D.-E. Lesemann[2]
[1]*Department of Plant Science, Obafemi Awolowo University, Ile-Ife, Nigeria;* [2]*Biologische Bundesanstalt für Land-und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, D-38104 Braunschweig, Germany; *Author for correspondence (Phone: +49 531 2993750; Fax: +49 531 2993006; E-mail: r.koenig@bba.de)*

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Abstract

Field-grown tobacco plants in Nigeria showing chlorotic mottle and marginal veinbanding on the leaves apparently contained several viruses. One of them proved to be a new *Tobamovirus* for which we suggest the name *Nigerian tobacco latent virus* (NTLV), because it did not produce systemic symptoms on various cultivars of *Nicotiana tabacum*. Sequence analyses of the coat and movement protein genes and their translation products, as well as serological studies, revealed that NTLV is only distantly related to known *Tobamoviruses* from which it also differs in host range and symptomatology. Its closest relationship was found to *Tobacco mild green mosaic virus* (TMGMV). The percentages of amino acid sequence identity amounted to 73% for the coat proteins and to 64% for the movement proteins of the two viruses. The total sequence of 1415 nucleotides analysed share 63% identity with the corresponding region of TMGMV. In the immunoelectron microscopical decoration test using antisera at a dilution of 1 : 50, reactions of NTLV were observed only with its own antiserum and one out of two antisera to TMGMV. An antiserum to NTLV diluted 1 : 2 failed to react with TMGMV. NTLV induces the formation of characteristic inclusions in infected cells.

Introduction

Tobacco-growing is encouraged by the tobacco-processing industry in Nigeria. Field symptoms suggest frequent virus infections in this crop, but so far the only virus from tobacco identified in Nigeria is *Pepper veinal mottle virus* (Ladipo and Roberts, 1979). Plants on one field which showed chlorotic mottle and short veinbanding at the margins of the leaves were studied in more detail. Transmission experiments suggested that they contained several viruses. One of them proved to be a new *Tobamovirus* which will be described in this paper.

Materials and methods

Virus isolation and host range studies

For the initial virus isolation, young leaves from field-grown tobacco were ground in 0.03 M sodium phosphate buffer, pH 8.0. The homogenate was rubbed on carborundum-dusted leaves of several *Nicotiana* species. After separation from other viruses (see Results), the *Tobamovirus* was maintained in *Nicotiana benthamiana*. For host range studies, five plants of each test species were inoculated mechanically. Retrieval tests from symptomless test plants were carried out from inoculated leaves one week after inoculation and from top leaves one month after inoculation. *N. glutinosa* was used as an indicator plant.

Virus purification and antiserum production

The *Tobamovirus* was purified from infected *N. benthamiana* (Hull et al., 1976), with modifications described by Koenig et al. (1984). A rabbit was immunized by two intramuscular injections spaced one week apart with 2 mg virus each that was emulsified in 2 ml Freund's complete and incomplete adjuvant, respectively. Bleedings were taken at 2-week intervals.

Electron microscopy

For visualising virus particles, crude plant extracts were incubated for 5 min with Pioloform-carbon coated copper grids. Negative staining was done with 1% aqueous uranyl acetate. For assessing their serological reactivity, adsorbed particles were decorated with antisera diluted 1 : 50 (Milne, 1984). Decoration titers were determined with 1 : 2 serial dilutions of antisera starting with a dilution of 1 : 50. The cytopathology of infected *N. benthamiana* was studied on ultrathin sections made after fixation of leaf tissues with glutaraldehyde followed by osmium tetroxide and embedding in Epon (Koenig and Lesemann, 1985).

Sequence analyses

Immunocapture reverse transcription PCR was done with sap from infected *N. benthamiana* as described for *Beet necrotic yellow vein virus* (Koenig et al., 1995). The primers P1 and P2 were derived from areas in the nucleotide sequence of *Pepper mild mottle virus* (PMMoV) RNA (Accession No. M81413) which are highly conserved in other *Tobamoviruses*. P1 corresponds to nucleotides 4848–4877 and P2 is complementary to nucleotides 6234–6254 of PMMov RNA. P2 was used for cDNA synthesis. The PCR product obtained with P1 and P2 was purified using the Jetsorb Gel Extraction Kit (Genomed), cloned into the pGEM-T vector (Promega) and sequenced by a commercial company (MWG-Biotech, D85560 Ebersberg/Germany), which provided more than 1000 nucleotides in both directions for one clone. Sequences were analysed by means of the LINEUP/PILEUP programs of the UWGCG software version 8 (Devereux et al., 1984) and trees were generated by the program DNAMAN (Lynnon Bio/Soft).

Results

Biological properties

Sap from young leaves of field-grown tobacco plants was rubbed on several *Nicotiana* species. Systemic symptoms were produced on *N. debneyi*, *N. megalosiphon*, *N. occidentalis* (wild type) and *N. repanda*. The latter three plant species also developed local lesions. Apparently, several viruses were present, because inoculum prepared from systemically infected *N. megalosiphon* or *N. repanda* induced similar systemic symptoms in these hosts but no local lesions. A virus that produced only local necrotic lesions in *N. megalosiphon* and *N. repanda* and a systemic mosaic in *N. occidentalis* was isolated from systemically infected leaves of the originally inoculated plants of *N. occidentalis.* These leaves had been dehydrated over calcium chloride in the cold. Electron microscopy revealed the presence of abundant ca. 300 nm long *Tobamovirus*-like particles in all three plant species. The properties of this virus which are described in the following sections indicate that it is a new *Tobamovirus* for which we propose the name *Nigerian tobacco latent virus* (NTLV), because it did not produce systemic symptoms on various cultivars of *N. tabacum*. The host plant reactions of NTLV are summarized in Table 1.

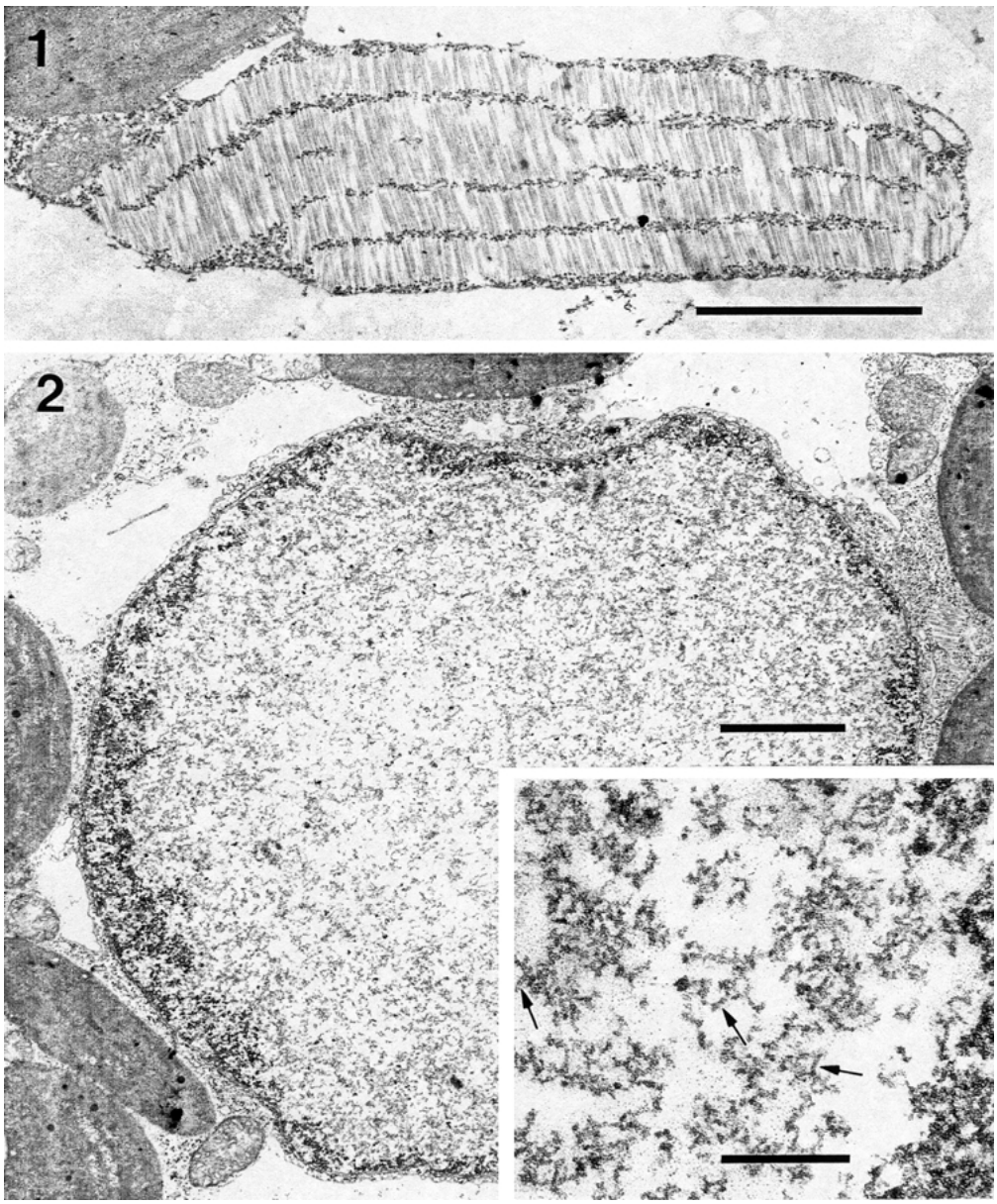
Table 1. Reactions of test plants to NTLV

Plant species	Reaction
Antirrhinum majus L.	Symptomless infection of inoculated leaves only
Gomphrena globosa L.	Symptomless infection of inoculated and top leaves
Chenopodium amaranticolor Coste & Reyn.	Few and scattered local lesions, no systemic symptoms
Nicotiana benthamiana Domin.	Broad yellow areas and some leaf cupping
N. clevelandii Gray	Mild mottle
N. glutinosa L.	Small local necrotic lesions
N. megalosiphon Heurck & Mueller	Necrotic local lesions
N. occidentalis Wheeler (wild type)	Systemic mosaic and veinbanding
N. repanda Willd. Ex Lehm	Necrotic lesions
N. tabacum L.	
cv. Havana 425	Symptomless infection of inoculated and newly developing leaves
cv. NC 95	Local broken rings, symptomless infection of newly developing leaves
cv. Samsun NN	Necrotic local lesions and some white rings, no systemic infection
cv. T.I. 787	Local broken rings, no systemic infection
cv. Turkish	Symptomless infection of inoculated and newly developing leaves
cv. Xanthi nc	Necrotic local lesions, no systemic infection

No infections were observed on *Arachis hypogaea*, *Datura stramonium*, *Dolichos biflorus*, *Physalis peruviana*, *Lycopersicon esculentum*, *N. rustica* and *Solanum melongena* cv. Ex Jos. In crude sap, NTLV remained infectious after heating for 10 min at 85 °C, but not at 90 °C and after dilution to 10^{-3} but not to 10^{-4}.

Cytopathology

In infected cells of *N. benthamiana* massive cytoplasmic inclusions consisting of stacked plate-like layers of virus particles were observed (Figure 1). They are typical for many *Tobamoviruses*. Each layer was a lateral aggregate of the rod-like particles with the particle ends



Figures 1 and 2. Cytopathology of NTLV-infected *N. benthamiana* cells. 1. Stacked plate-like aggregate of virus particles; 2. Enlarged nucleus containing homogenous low-contrast material consisting of accumulated short virus-like particles (arrows in enlarged inset). Bars equal 2 μm and 300 nm on the main figure and the inset, respectively.

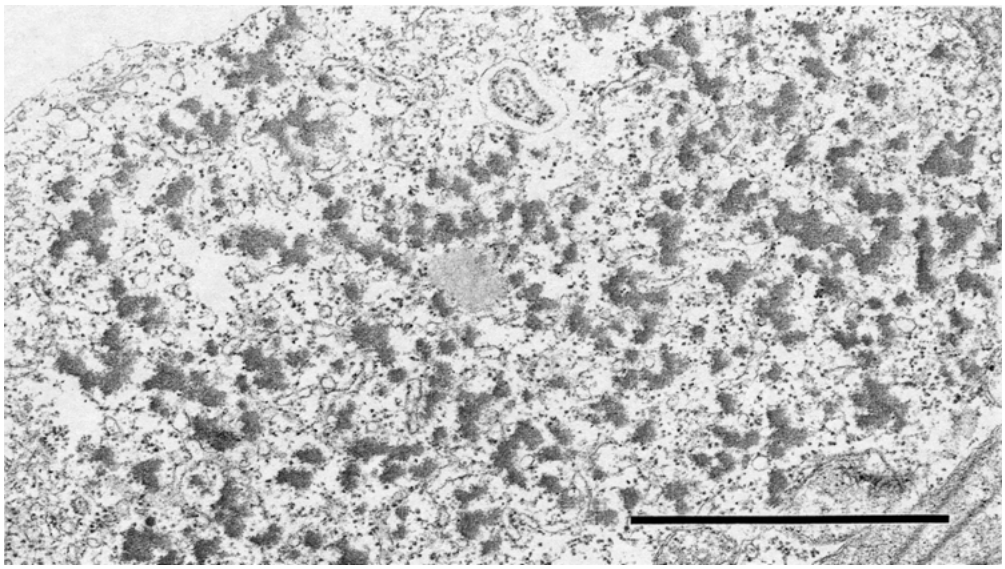


Figure 3. Cytoplasmic accumulation of hypertrophied endoplasmic reticulum mixed with scattered dark staining material in NTLV-infected *N. benthamiana* cells. Bar equals 2 μm.

in register. Presumably, due to fixation artifacts, the plate-like layers were also found in disordered arrangements. The nuclei of infected cells were often enlarged and showed voluminous uniformly-structured central regions of low contrast (Figure 2) which appeared to contain loosely and irregularly aggregated very short rod-like particles (Figure 2, enlarged inset). In addition, accumulations of hypertrophied endoplasmic reticulum membranes and other cytoplasmic material were observed which contained irregularly shaped clusters of dark staining material in scattered distribution (Figure 3). In infected cells, low numbers of ca. 50–100 nm measuring flask-like vesicles were also recorded at the tonoplast membranes as buddings into the vacuoles from the cytoplasmic compartment (not shown).

Serology

The serological affinities of NTLV were studied by means of the immunoelectron microscopical decoration test (Tables 2 and 3). In a preliminary screening test, using antisera to 14 different *Tobamoviruses* at a dilution of 1 : 50, NTLV reacted strongly only with its own antiserum and one out of two antisera to *Tobacco mild green mosaic virus* (TMGMV). Determination of the decoration titers revealed that the second TMGMV antiserum at

Table 2. Reactivity of NTLV from dried leaf tissue with 1 : 50 diluted antisera to 14 different members of the genus *Tobamovirus* in the immunoelectron microscopical decoration test

Antisera to *Tobamoviruses*[1]	Decoration intensity[2]
Bell pepper mottle	−
Cucumber green mottle mosaic	−
Frangipani mosaic	−
Maracuja mosaic	−
NTLV	+++
Odontoglossum ringspot	−
Paprika mild mottle	−
Pepper mild mottle	−
Ribgrass mosaic (EM-AS45)	−
Ribgrass mosaic (Streptocarpus flower break EM-AS1496)	+
Sammon's Opuntia	−
Sunnhemp mosaic	−
TMGMV (DSMZ AS-0181)	++−+++
TMGMV (*Para-tobacco mosaic virus*, Wetter)	−
Tobacco mosaic (TMV vulgare, Adam)	−
Tobacco mosaic (DSMZ PV-AS0041)	−
Tobacco mosaic Ohio III (DSMZ PV-AS0043)	+
Tomato mosaic	−

[1]Antisera were from the stock of the Institut für Pflanzenvirologie, Mikrobiologie und Biologische Sicherheit or from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DMSZ).

[2]Decoration intensity assessed as: − = undecorated; + = weak; ++ = medium; +++ = strong.

Table 3. Homologous and heterologous decoration titers of antisera to NTLV and TMGMV

Antisera	Antigens	
	NTLV	TMGMV[2]
NTLV	1600[1]	<2
TMGMV (DSMZ AS-0181)	800	1600
TMGMV (*Para-tobacco mosaic virus*, Wetter)	5	200

[1]Reciprocal dilution endpoints of the decoration reaction.
[2]Similar results with TMGMV sources from *N. glauca* (U2), *N. tabacum* (*Para-tobacco mosaic virus*), *Capsicum annum*, *Impatiens* ('New Guinea Hybrid') and *Petunia*.

a lower dilution also reacted weakly with NTLV (Table 3). An antiserum to NTLV diluted 1 : 2 did not react with various sources of TMGMV (Table 3).

Sequence analyses

The primers P1 and P2, which were derived from highly conserved sequences upstream of the movement protein gene and downstream of the coat protein gene of PMMoV, respectively, allowed us to amplify a stretch of 1415 nucleotides that contained the entire movement and coat protein genes of NTLV and short additional sequences upstream and downstream of these genes. Analyses of the nucleotide sequences of the movement and coat protein genes and of the deduced amino acid sequences of their translation products (Figure 4) suggested that NTLV is only distantly related to all known *Tobamoviruses* and corroborates the observation made by means of serology that it has its closest relationships to TMGMV. Very similar groupings were obtained for the movement proteins and for the coat proteins of *Tobamoviruses* in trees which were based either on the neighbour joining method of Saito and Nei (1987) (Figure 4, left side trees) or on percentages of sequence identities (Figure 4, right side trees). Both approaches revealed that both the movement and the coat protein of NTLV have their closest relationships to the corresponding proteins of TMGMV. The percentages of amino acid sequence identity amounted to 64% for the movement proteins and to 73% for the coat proteins of the two viruses (Figure 4). The total sequence of 1415 nucleotides analysed shares 63% sequence identity with the corresponding region of TMGMV RNA. The genebank accession number for this partial NTLV sequence is AY137775.

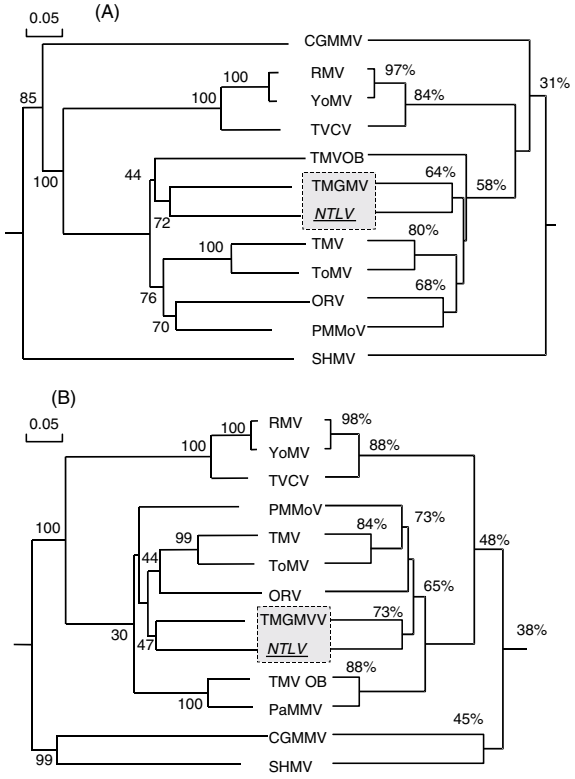


Figure 4. Trees based on neighbour joining analyses (Saitou and Nei, 1987) (left side) and on percentages of amino acid sequence identity (right side) for the movement proteins (A) and the coat proteins (B) of NTLV and those *Tobamoviruses* for which accession numbers have been listed by Lewandowski (2000). The numbers on the branches on the left side indicate percent boot strap scores in 1000 trials. The length of the branches on the left site of the diagrams can be estimated by means of the scale bar given above each figure.

Discussion

NTLV is probably not a major cause for the tobacco disease observed in Nigeria, because systemic infections in several cultivars of *N. tabacum* were symptomless. The biological, cytopathological, serological and molecular properties of NTLV indicate that it is distinct from all known *Tobamoviruses*. In host range and symptomatology, it differs from all other *Tobamoviruses* including a strain of *Tobacco mosaic virus* (TMV) from pepper, the only *Tobamovirus* described in Nigeria before (Igwegbe, 1983). Whereas the pepper strain of TMV induces systemic mosaic without local lesions in *N. megalosiphon*, NTLV induces only necrotic local lesions in this host.

In *N. glutinosa* and *D. stramonium* the pepper strain induces necrotic local lesions and lethal systemic necrosis, whereas NTLV induces necrotic local lesions only in *N. glutinosa* and fails to infect *D. stramonium*. *Tobamoviruses* isolated by Burgyan et al. (1978) from tobacco, tomato, pepper and *S. dulcamara* all induced necrotic local lesions in *D. stramonium*. NTLV, like the para-tobacco mosaic virus strain of TMGMV isolated from field-grown tobacco in Germany (Wetter, 1980), fails to infect *L. esculentum*.

Cytological studies revealed particle aggregates and nuclear accumulations of short virus-like particles as described for many other *Tobamoviruses* (Francki et al., 1985), but unlike several other *Tobamoviruses* (Francki et al., 1985; Granett and Shalla, 1970), NLTV failed to form particles in the chloroplasts. Small membrane-attached vesicles are typically induced by members of the sindbis-like superfamily of single stranded RNA viruses including several *Tobamoviruses*. They may represent the 'replicative complexes' postulated as structural basis for the RNA replication (Lesemann, 1991).

A distinct property of NTLV appears to be the formation of scattered patches of dark staining material in cytoplasmic accumulations. NTLV fails to produce inclusions resembling the classical X-bodies induced by TMV. These X-bodies are formed from bundles of tubular structures (Esau and Cronshaw, 1967) and have been proven to contain accumulations of the viral non-structural 126 kDa protein, a putative component of the RNA replicase (Hills et al., 1987; Saito et al., 1987; Wijdeveld et al., 1989; 1992). With other *Tobamoviruses* amorphous structures have been described (Esau and Cronshaw, 1967; Francki et al., 1985) and also plate-like arrangements have been observed (Lesemann, unpublished observations) which may be equivalent to the X-bodies. The dark patchy structures induced by NTLV might represent a new type of X-body-like cellular inclusions.

Sequence analyses indicate that NTLV has its closest relationships to TMGMV, but these relationships are far more distant than, for instance, those between TMV and *Tomato mosaic virus* (Figure 4) which are considered to represent different virus species (Lewandowski, 2000). Serology also suggested that NTLV is more closely related to TMGMV than to any other *Tobamovirus*. However, these relationships likewise appeared to be rather distant or even absent with two of the three antisera tested, i.e. one out of two to TMGMV and one antiserum to NTLV, respectively (Table 3). It therefore seems to be justified to consider NTLV as a distinct virus species.

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