Cynodon Chlorotic Streak Virus, a Previously Undescribed Plant Rhabdovirus Infecting Bermuda Grass and Maize in the Mediterranean Area

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ABSTRACT

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A virus first observed on maize in southern Morocco in 1974 was subsequently found to be endemic in Bermuda grass (*Cynodon dactylon*) in Morocco, and occurs in Bermuda grass in France, Spain, Tunisia, and Jordan. The virus causes stunting and chlorotic streaking in both Bermuda grass and maize. The virus was not transmitted mechanically or by two cicadellid leafhoppers commonly found on *C. dactylon*, but was transmitted by the delphacid planthopper *Toya propinqua*. Virus particles in negatively stained leaf dip preparations were mainly bullet-shaped and measured approximately 72 × 240 nm. In ultrathin sections, virus particles measured 80 × 280 nm and accumulated in the perinuclear space. The virus was partially purified by differential centrifugation, and an antiserum was produced that had a homologous titer of 1/128 against undiluted infected sap in immunodiffusion tests. The virus was also detected in field samples of Bermuda grass and maize using enzyme immune assay (EIA). The virus was serologically unrelated to maize mosaic virus (MMV), cereal chlorotic mottle virus (CCMV), maize sterile stunt virus (MSSV), barley yellow striate mosaic virus (BYSMV), or Festuca leaf streak virus. The name Cynodon chlorotic streak virus (CCSV) is proposed for the virus, which shares with viruses belonging to the proposed subdivision II of the plant rhabdovirus group, the property of perinuclear assembly.

Rhabdoviruslike particles were found to be associated with a chlorotic streak disease of maize and was first observed in Morocco in 1974 (13). The virus was not transmitted mechanically and was not identified. Subsequently, similar symptoms were observed in Bermuda grass (*Cynodon dactylon* L.), and viruslike particles of similar morphology and dimensions were associated with the disease in Bermuda grass. Surveys revealed that these symptoms, and associated rhabdoviruslike particles, occurred endemically in Bermuda grass in Morocco, and also occurred in maize growing in the vicinity of infected Bermuda grass. This study was undertaken to determine the identity of the virus, hereafter referred to as Cynodon chlorotic streak virus (CCSV), its mode of transmission, and relationship to previously described plant rhabdoviruses infecting Gramineae.

MATERIALS AND METHODS

Virus source and culture. The original virus source consisted of naturally infected *C. dactylon*. The source plants showed typical chlorotic streak symptoms, contained rhabdoviruslike particles, and had no symptoms of the mycoplasmalike disease, similar to Bermuda grass yellow leaf (1), that occurs commonly on this plant in Morocco (B. E. L. Lockhart and N. Khaless, *unpublished*). No viruslike particles other than rhabdoviruslike particles were observed in negatively stained leaf dip preparations. Following identification of the planthopper vector, the virus was transmitted by planthopper from the original source plants to healthy *C. dactylon* grown from seed collected from healthy plants.

Virus distribution. Samples of Bermuda grass and maize showing chlorotic streak symptoms were collected at numerous locations throughout Morocco during 1981–1984. Initially, samples were checked by electron microscopic examination of negatively stained leaf dip preparations for the presence of rhabdoviruslike particles. After production of an antiserum specific to CCSV, identity of the virus in infected samples was confirmed by immunodiffusion tests or enzyme immune assays (EIA) in addition to routine electron microscopic examination. Samples of Bermuda grass showing chlorotic streak symptoms were also collected in northern Tunisia, in the Jordan Valley of Jordan, in southern Spain, and in the vicinity of Avignon, France. These samples were returned to Morocco for virus identification by electron microscopy and serology.

Mechanical transmission. Inoculum was prepared by grinding young infected *Cynodon* leaf tissue in cold 1% K_2HPO_4 containing 0.2% 2-mercaptoethanol. Carborundum-dusted healthy seedlings of the following test plants were inoculated mechanically with the crude extract: *C. dactylon*, *Zea mays* 'Golden Cross Bantam,' *Hordeum vulgare* L. 'NK 38,' *Avena sativa* L., and *Sorghum bicolor* × *S. sudanense* 'Trudan.'

Insect transmission. Two cicadellid leafhoppers, *Exitianus capicola* Stall. and *Psammotettix* sp., and a delphacid planthopper, *Toya propinqua* (Fieber) were used for virus transmission tests. These three species occur commonly on Bermuda grass in Morocco, and were also found on maize in the vicinity of infected Bermuda grass. All three species were reared on healthy Bermuda grass. They were allowed an acquisition access period of 3–12 days on infected Bermuda grass, and then transferred to healthy test seedlings of Bermuda grass or maize until they died. All experiments were done by mass transfer. Groups of 15–30 individuals were caged on groups of 10–20 test seedlings during the inoculation access period. No attempts were made to determine minimum acquisition access period, latent period, efficiency of transmission, or any other aspects of vector-virus relationships.

Partial virus purification. CCSV was partially purified from young CCSV-infected *C. dactylon* leaf tissue. Initial tests using Celite clarification (10,15) gave unsatisfactory results. After initial tests on extraction and clarification methods, the following procedure was adopted: fresh tissue was extracted in four volumes

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(w/v) of cold 0.1 M sodium citrate, pH 6.5, containing 10% sucrose, 0.2% Na_2SO_3 and 4% activated charcoal. After filtration, the extract was acidified to pH 5.0 by dropwise addition of glacial acetic acid, stored at 4 C for 2–4 hr, and clarified by centrifugation at 17,400 *g* for 20 min. The virus was concentrated by ultracentrifugation at 91,500 *g* for 30 min and the resulting pellet resuspended in 0.01 M phosphate buffer, pH 7.2, containing 10% sucrose and 0.85% NaCl. The suspension was centrifuged at 107,000 *g* for 45 min on a continuous 12–50% sucrose gradient prepared in 0.01 M phosphate, pH 7.2, containing 0.85% NaCl. The virus-containing zone was collected and reconcentrated by centrifugation at 139,000 *g* for 30 min. The final pellet, resuspended in the same buffer, constituted the partially purified virus suspension.

Electron microscopy and histopathology. Leaf dip and partially purified preparations were negatively stained in 1.5% phosphotungstic acid, pH 7.0 (PTA) or in 2% ammonium molybdate, pH 7.0 (AM). For histopathological studies, leaf tissue of *Cynodon* was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8, and postfixed in 2% osmium tetroxide. After dehydration in an ethanol series, the tissue was embedded in Epon 812 resin. Thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate.

Serology. Antisera were prepared in rabbits by injection of partially purified virus suspensions emulsified in Freund's incomplete adjuvant. Animals were given four intramuscular and four subcutaneous injections, alternating at biweekly intervals. Blood was collected 2 wk after the final injection. Immunodiffusion tests were done in 0.85% agarose, 0.1% NaN_3, prepared in distilled water. Antiserum titer was determined by dilution in 5% bovine serum albumin in 0.05 M tris-HCl, pH 7.2, containing 0.85% NaCl (17). Crude undiluted plant sap was used as antigen without prior treatment by sonication or detergents. Antisera to Venezuelan (12) and U.S. (2) isolates of MMV were provided by R. Lastra and R. E. Gingery, respectively. Antiserum to Italian BYSMV (4) was obtained from M. Conti. Antiserum to a Moroccan BYSMV isolate (*unpublished*) was prepared by a purification and immunization procedure described for tomato vein-yellowing virus (TVYV) (5). Serological tests with CCMV (6), MSSV (7), and Festuca leaf streak mosaic rhabdovirus (14) antisera were done by R. S. Greber. Microplate enzyme immune assays were done by the double antibody sandwich (DAS) method (3). Polystyrene plates were coated with 1.0 μg/ml of purified anti-CCSV γ-globulin. Leaf samples were added at a 1:10 (w/v) dilution in phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 and 2% polyvinylpyrrolidone, MW 40,000 (3). Alkaline phosphate conjugate was used at a 1/500 dilution. Results were determined spectrophotometrically at 405 nm by using a Dynatech microplate reader.

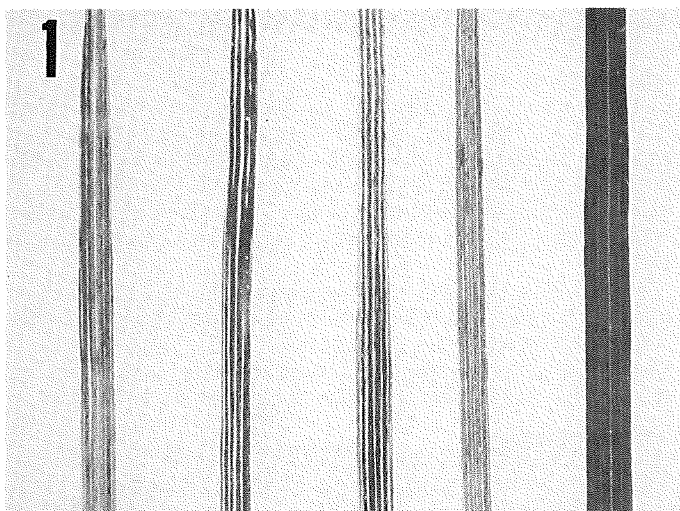


Fig. 1. Symptoms of Cynodon chlorotic streak virus (CCSV) infection in naturally infected *Cynodon dactylon*. Healthy leaf is at extreme right.

RESULTS

Virus distribution and symptoms. In surveys conducted in Morocco during 1981–1984, infection by CCSV was identified on the basis of symptoms, and confirmed in all cases by electron microscopy and immunodiffusion tests or EIA assay. The virus was found to be endemic on *C. dactylon*, and to occur less frequently on maize. Occurrence of the virus in cultivated maize was invariably associated with the presence of infected Bermuda grass within the field or along the border. The virus was also identified by symptomatology, electron microscopy, and serology in samples of Bermuda grass collected in southern France, northern Tunisia, and in the Jordan Valley of Jordan. Symptoms in all cases were identical to those observed on Bermuda grass in Morocco. Symptoms, consisting of narrow longitudinal chlorotic streaks, were similar in *Cynodon* (Fig. 1) and in maize (Fig. 2). Maize was sometimes found doubly infected with CCSV and maize dwarf mosaic virus (MDMV), which is the virus that occurs most commonly on maize in Morocco (13).

Mechanical transmission. CCSV was not transmitted by mechanical inoculation from infected *C. dactylon* to healthy *C. dactylon*, Golden Cross Bantam corn, barley, oats, or sudangrass.

Insect transmission. CCSV was not transmitted from infected to healthy *Cynodon* by either *Exitianus capicola* or *Psammotettix* sp., but was transmitted to *Cynodon* and maize by *Toya propinqua*. In addition to transmitting CCSV experimentally, adults and nymphs of *T. propinqua*, collected from naturally infected *C. dactylon* and caged directly on healthy *C. dactylon* and maize, transmitted CCSV to these plants. In all tests, CCSV transmission was confirmed by electron microscopy and immunodiffusion tests.

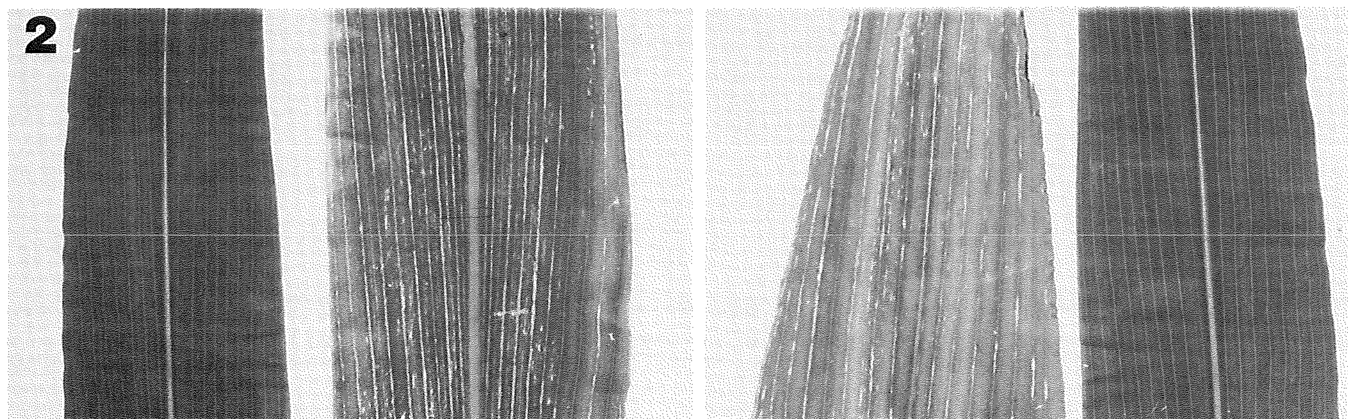


Fig. 2. Symptoms of Cynodon chlorotic streak virus (CCSV) infection in *Zea mays* resulting from transmission of CCSV by *Toya propinqua* from *Cynodon dactylon*. Healthy leaves at right and left, infected leaves in center.

Partial purification. As determined by electron microscopy at each step in the purification sequence, 0.1 M citrate buffer, pH 6.5, was the most suitable extraction buffer, and the addition of 10% sucrose greatly reduced particle disruption during purification. The addition of 4% activated charcoal reduced the amount of pigmented material in the final suspension, but appeared to have no effect on particle numbers or integrity. Clarification by filtration through Celite, which has been used for purification of several other plant rhabdoviruses, including TVYV (5), was found to be unsuitable for CCSV. Clarification by acidification was found superior both for particle yield and integrity. The final partially purified suspension was considerably contaminated with host material, and separation of the virus from much of this contaminating material was found to be impractical on sucrose gradients.

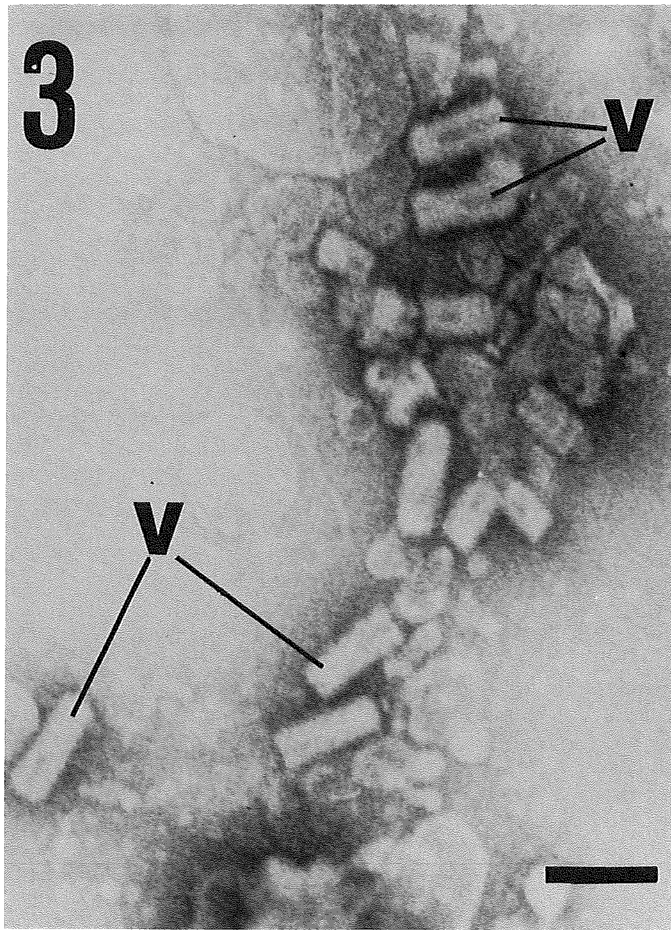
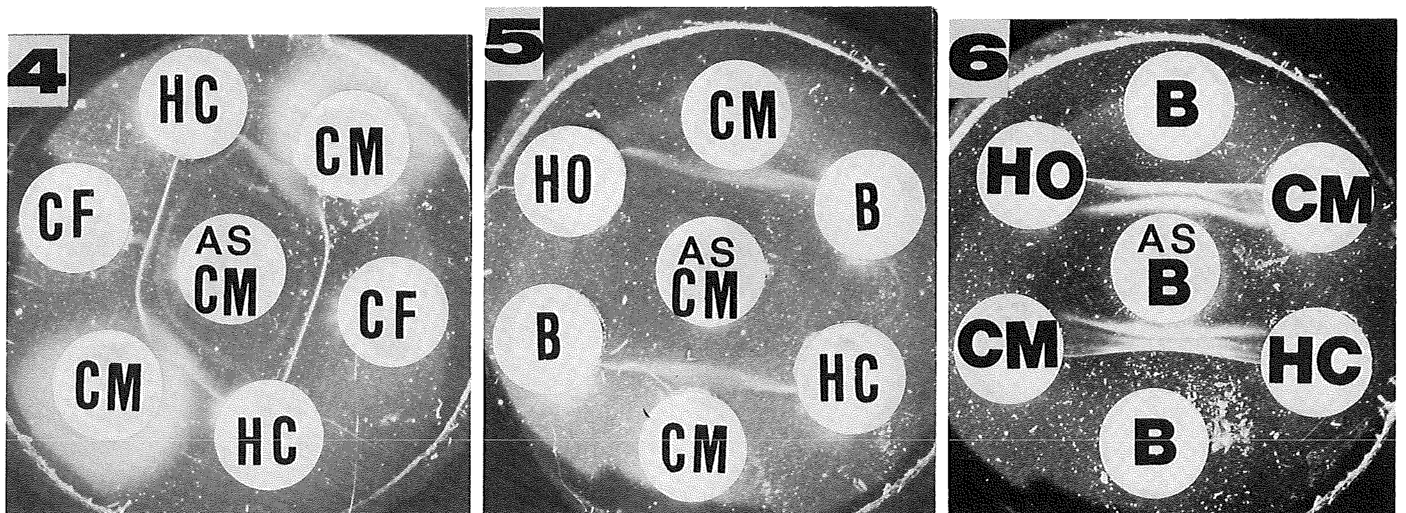


Fig. 3. Bullet-shaped particles (V), some broken, of Cynodon chlorotic streak virus (CCSV) in an unfixed leaf dip preparation of *Cynodon dactylon*. Negatively stained with 1.5% sodium phosphotungstate, pH 7.0. Scale bar represents 300 nm.

Serology. CCSV usually produced a single precipitin line with homologous antiserum in immunodiffusion tests in 0.85% agarose in distilled water (Fig. 5). A second specific precipitin line (Fig. 4) was obtained with some samples, and also depended on the antiserum concentration used. Satisfactory results were obtained with untreated plant sap, and no ultrasonic or detergent treatment (6,16) was used. The CCSV antiserum had a homologous titer of 1/128 in gel diffusion tests using undiluted sap from infected *C. dactylon* as antigen. Moroccan and French isolates (Fig. 4) and Spanish isolates of CCSV produced confluent precipitin lines. CCSV did not react with antisera to MMV, CCSV, Festuca leaf streak mosaic rhabdovirus, Italian BYSMV, or Moroccan BYSMV. Moroccan BYSMV antigen also did not react with CCSV antiserum (Figs. 5 and 6). CCSV was readily detected in infected Bermuda grass leaf tissue by DAS-EIA assay. Under the conditions described above, 405 nm absorption values for CCSV-infected tissue ranged from 1.00 to 1.68, while corresponding values for healthy tissue were from 0.03 to 0.09.

Electron microscopy and histopathology. In leaf dip or partially purified preparations, CCSV was stable in both PTA and AM without prior fixation. Numerous particles were observed in leaf dips, and these virus particles frequently occurred in membrane-bound groups. The majority of particles appeared bullet-shaped (Fig. 3), but bacilliform particles were occasionally observed. In leaf dips negatively stained with PTA, bullet-shaped particles measured approximately 72 × 240 nm. Based on frequency of particles in leaf dip preparations of equal sap concentration, CCSV occurs in considerably higher concentration in *Cynodon* than in maize.

In ultrathin sections, the virus particles appeared to bud from the nucleus. They appeared to assemble on the surface of the inner lamella of the nuclear membrane and accumulate in the perinuclear space (Fig. 7). Aggregates of particles occurring in the cytoplasm were always surrounded by a membrane, probably originating



Figs. 4–6. Homologous and heterologous immunodiffusion reactions of Cynodon chlorotic streak virus (CCSV) and barley yellow striate mosaic virus (BYSMV, Moroccan isolate) antigens and their respective antisera. Undiluted sap from infected leaf tissue was used as the viral antigen in all cases. Immunodiffusion medium is 0.85% agarose and 0.2% NaN_3 in distilled water. **4,** Reaction of CCSV isolates from Morocco (CM) and France (CF) with antiserum to Moroccan CCSV (AS-CM). HC = undiluted sap from healthy *Cynodon dactylon*. **5,** Reaction of Moroccan isolates of CCSV (CM) and BYSMV (B) with antiserum to Moroccan CCSV (AS-CM). BYSMV is in undiluted sap from infected oats. HO = undiluted sap from healthy oats. HC = undiluted sap from healthy *C. dactylon*. **6,** Reaction of Moroccan CCSV (CM) and BYSMV (B) isolates with antiserum to Moroccan BYSMV (AS-B). Healthy controls as in Fig. 5.

from the outer lamella of the nuclear membrane. Particles in ultrathin sections from the virus-infected plants measured 80 × 280 nm.

DISCUSSION

Although CCSV was first found infecting maize, it appears to be primarily a virus of *C. dactylon* and only secondarily a virus of maize. The virus is probably widely distributed and is a potential pathogen of maize in the Mediterranean area. The virus shares some properties with MMV (8), which has been reported from South (11,12) and North America (2), Hawaii, Mauritius, and elsewhere (11). Both MMV and CCSV are planthopper-transmitted, both accumulate in the perinuclear space, and both viruses are similar in size and morphology (8). Separate serological tests conducted in Venezuela, Australia (R. Greber, *personal communication*), and Morocco all failed to show any relationship between the two viruses, however. It is not known whether CCSV can be transmitted by *Peregrinus maidis*, the principal vector of MMV (8), or whether *T. propinqua* is capable of transmitting MMV. In early surveys, *P. maidis* was not found in Morocco (13). This delphacid planthopper has recently been found on maize in southern Morocco, although in low numbers (B. E. L. Lockhart, *unpublished*). *Toya propinqua*, on the other hand, breeds prolifically on Bermuda grass, and occurs in high numbers, especially from January through May (B. E. L. Lockhart, *unpublished*).

Based on host range, symptomatology, histopathology, vector specificity, and serological properties, CCSV appears to be distinct from previously described members of the plant rhabdovirus group. The virus is morphologically distinct from and serologically unrelated to BYSMV, which occurs in Morocco on wheat, barley and oats, and is also transmitted by *T. propinqua* (*unpublished*). Like CCSV, BYSMV (= wheat chlorotic streak mosaic virus) occurs in France (19). The possible relationship of CCSV to a planthopper-transmitted rhabdovirus occurring on maize, wheat, barley, and other Gramineae in Iran (9), and to a rhabdovirus observed in ultrathin sections in infected maize in Spain (18), was not determined. The Iranian rhabdovirus and CCSV differ in particle size, and in limited tests, CCSV was not transmitted by *T. propinqua* to either wheat or barley. The cellular site of accumulation of CCSV indicates an affinity with other plant rhabdoviruses of the proposed subdivision II (16). Further chemical and biochemical characterization of CCSV will be required in order to confirm this relationship. It is evident that the lack of availability of antigen and of high-titered antisera, and a paucity of data on chemical properties, limit the study of relationships between viruses in this group.

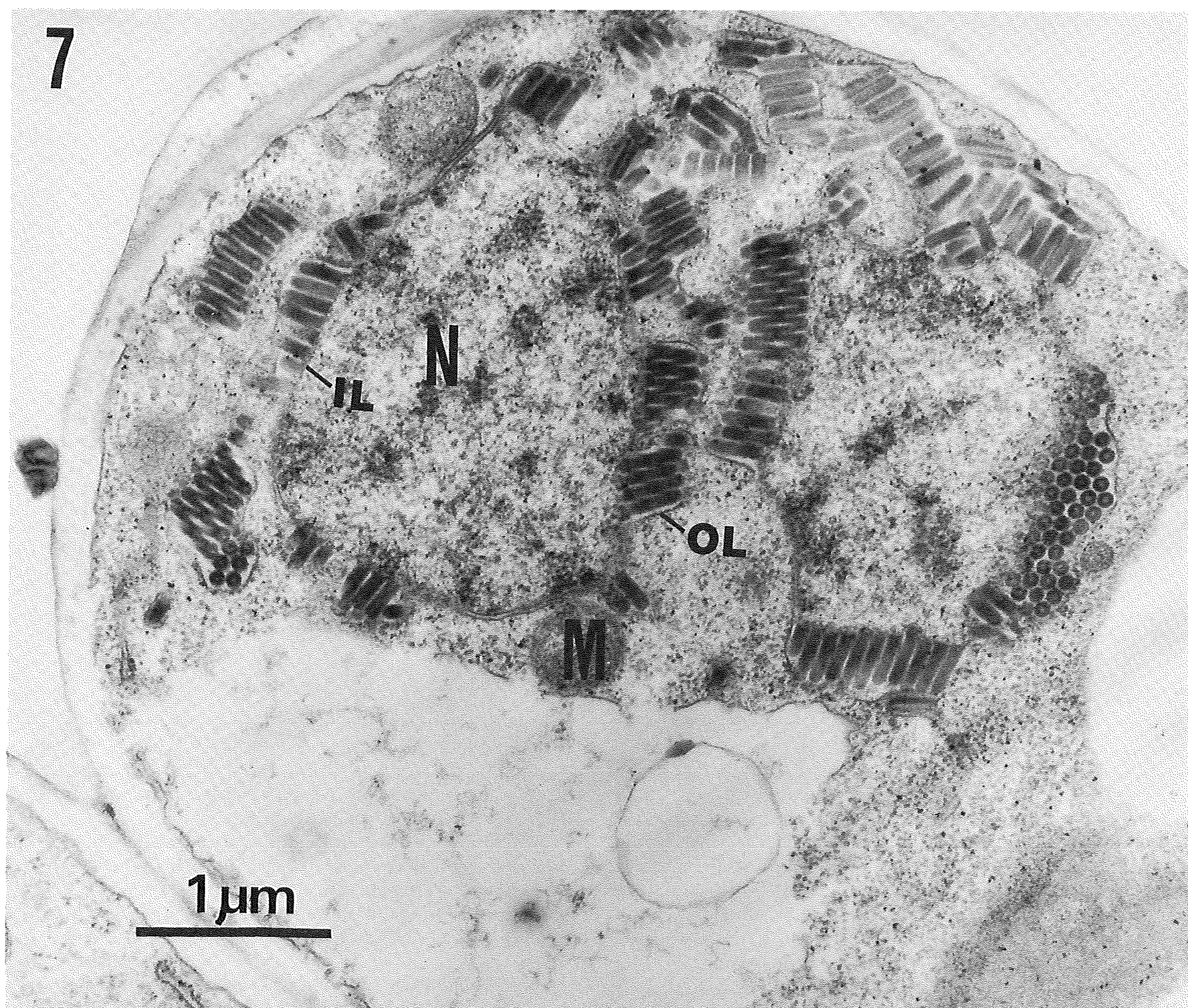


Fig. 7. Intracellular appearance of Cynodon chlorotic streak virus (CCSV) in a young leaf cell of *Cynodon dactylon*. N = nucleus, M = mitochondrion, IL = inner lamella, and OL = outer lamella.

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