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A reovirus in the brown planthopper, *Nilaparvata lugens*

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A new virus, belonging to the reovirus group, was found in an apparently healthy colony of the brown planthopper, *Nilaparvata lugens*, and was referred to as the *Nilaparvata lugens* reovirus (NLRV). The virus was found in the cytoplasm of the insect cells, sometimes associated with tubular structures, which is one of the characteristic features in tissues infected with reoviruses. The virus was purified by carbon tetrachloride clarification, polyethylene glycol precipitation, differential and CsCl equilibrium centrifugations. The virus has double-shelled particles approximately 65 nm in diameter, containing 10 genome segments of dsRNA. The electrophoretic profile of the dsRNA segments differed from those of viruses associated with rice planthoppers and leafhoppers. Seven proteins were detected in a purified preparation of the virus: four were associated with the core particle and three with the outer shell. A virus antigen was detected in individual insects by ELISA. The virus is retained after injection and is vertically transmitted to the offspring.

Introduction

The brown planthopper, *Nilaparvata lugens,* is the most serious pest of the rice plant in East and Southeast Asia, because of the severe damage it causes by sucking sap and transmission of viruses to the rice plant. The planthopper transmits two kinds of viruses, rice ragged stunt virus (RRSV), a member of the reovirus group, and rice grassy stunt virus, a member of the tenuivirus group. These viruses multiply in the planthopper but are not transmitted via the eggs (Hibino, 1989). During studies of planthopper- and leafhopper-borne viruses, we found virus-like particles in a colony of *N. lugens* and named them *Nilaparvata lugens* reovirus (NLRV). To study vector–virus interaction and insect immune reaction against commensals, the virus was purified and the proteins and nucleic acids were analysed. A serodiagnostic system was established to detect the viruses in individual insects using antiserum against the purified virus.

Methods

Insects. Two colonies of the brown planthopper, *N. lugens,* were maintained on rice seedlings under constant conditions of 25 °C and 16 h daily illumination. One, the Izumo colony was collected at Izumo, Shimane in the summer of 1987 and had been reared for about 25 generations. The other, the Chikugo colony, collected at Chikugo, Fukuoka in the summer of 1989, was obtained from Dr T. Wada of the Tropical Agriculture Research Center and had been reared for three generations.

Electron microscopy. Insect homogenates or purified preparations were negatively stained with 2% uranyl acetate and were studied under a Hitachi H-7000 electron microscope.

Insect intestines were fixed with 2·5% glutaraldehyde in 0·05 M-cacodylate buffer pH 7·4 for 1 h at 4 °C and post-fixed in 1% osmium tetroxide for 2 h at 4 °C. The samples were stained with 1% uranyl acetate and embedded in Epon 812. Thin sections were made with a Sorvall MT2-B ultramicrotome and stained with lead citrate.

Purification. The virus was purified according to the method of Omura *et al.* (1982) with some modifications. A partially purified preparation was obtained from frozen planthoppers by carbon tetrachloride clarification and polyethylene glycol precipitation. The suspension in 0·1 M-histidine buffer containing 0·01 M-$MgCl_2$, pH 7·0 (His-Mg) was layered on 10 to 40% (w/v) linear sucrose gradients in His-Mg and centrifuged for 80 min at 88000 *g* in a Hitachi RPS-27 rotor. The pellet was resuspended in His-Mg and CsCl was added to the suspension to a final concentration of 40% (w/v). The CsCl solution was centrifuged overnight at 190000 *g* in a Hitachi RPS-50-2 rotor. The zone containing virus particles was recovered and the virus was again centrifuged overnight at 190000 *g* in 40% CsCl. The virus was concentrated by ultracentrifugation and the final pellet was resuspended in His-Mg. Each step in the purification procedure was monitored by electron microscopy.

Virus injection. Purified preparations of the virus were injected into the thorax of fourth- to fifth-instar nymphs of virus-free *N. lugens* (Chikugo colony) using a glass capillary. The virus-injected insects were reared on rice seedlings and the resulting adults were allowed 7 days after injection to lay eggs in rice seedlings to produce an artificially virus-inoculated colony of planthoppers.

Serology. The BALB/c mice were immunized with purified virus by an intraperitoneal injection. Serological reactions were monitored by immunoelectron microscopy. An ELISA was carried out as described by Hibino & Kimura (1982).

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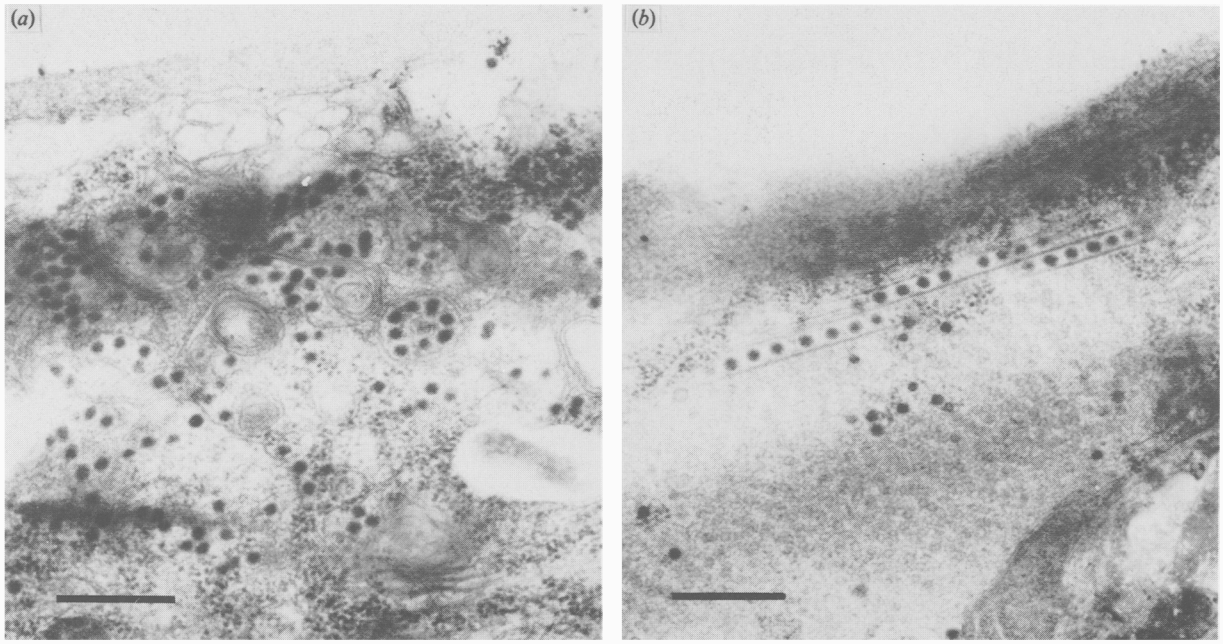

Fig. 1. Virus-like particles in thin sections of the intestine of *N. lugens*. Bar markers represent 500 nm.

Nucleic acid analysis. RNA was extracted from virus particles in purified preparations according to the method of Reddy & Black (1973). PAGE was as described by Omura *et al.* (1985*b*). Electrophoreses with 1% agarose gels were carried out to estimate the M_r of genomic RNA. Agarose–acrylamide composite gels (2% acrylamide and 0·5% agarose) were also used for nucleic acid analysis (Ishikawa *et al.*, 1989). Nucleic acids in the gels were digested by DNase I or RNase A, or were stained with acridine orange in the composite gel.

Protein analysis. The outer capsid from the virus particles was removed by adding $MgCl_2$ to the purified virus in His-Mg to a final concentration of 1·9 M (Hagiwara *et al.*, 1986). The suspension was ultracentrifuged for 20 min at 172 kPa (130000 *g*) using a Beckman Air Fuge. The core particles were recovered from the pellet, and the outer capsid proteins in the supernatant were precipitated using TCA.

Purified intact virus particles, core particles and the dissociated outer capsid proteins were processed as described by Omura *et al.* (1985*b*). The samples were electrophoresed in a 10% polyacrylamide gel (Laemmli, 1970).

Immunoblotting. Polypeptides were transferred from polyacrylamide gels to nitrocellulose sheets using a transfer blotting apparatus (Nippon Eido, NA-1512) and reacted with antiserum to intact virus particles or to core particles as described by Matsuoka *et al.* (1985).

Results

Electron microscopy

Reovirus-like particles (NLRV), approximately 65 nm in diameter, were found in negatively stained preparations of most planthoppers of the Izumo colony. On the other hand no particles of that description were observed in the Chikugo colony. Similar particles were observed in thin sections made from individual insects of the Izumo colony. They occurred in aggregates or were scattered throughout the cytoplasm (Fig. 1*a*). Some of them were surrounded by multi-membranous structures. Particles in tubular structures (Fig. 1*b*), were also found in the sections, a characteristic feature in plant and insect tissues infected with plant reoviruses (Milne & Lovisolo, 1977; Shikata, 1977; Omura *et al.*, 1985*a*).

Purification of virus particles

We usually used 50 to 100 g of insects. Each step in the purification procedure was monitored by electron microscopy. As NLRV did not form a clear band in sucrose density gradient centrifugation, we employed CsCl equilibrium centrifugation at the final step. The purified particles (Fig. 2*a*) showed a peak of u.v. absorbance at 260 nm and the ratio of u.v. absorbance at 260 and 280 nm was 1·307, suggesting that nucleoproteins had been purified. From 100 g of insects, about 1 ml of purified particle solution was obtained (A_{260} = 9·0). A faint band was also observed lower in the centrifuge tubes in the CsCl solution. When observed under the electron microscope, this band contained the core of the particles. This was ascertained by treating purified particles with 1·9 M-Mg^{2+}; they were stripped of their outer shells, revealing the core particles (Fig. 2*b*).

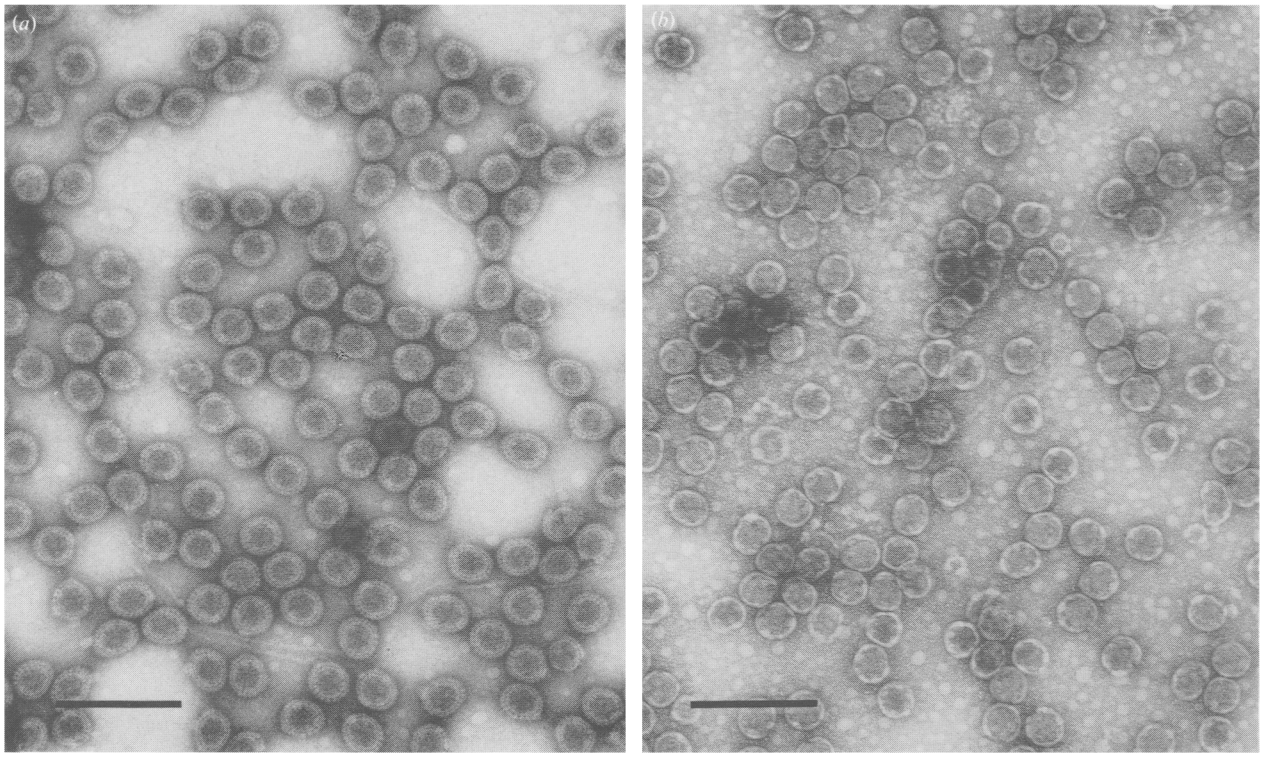

Fig. 2. Purified intact virus particles (*a*) and core particles (*b*) stained with 2% uranyl acetate. Bar markers represent 200 nm.

Nucleic acid analysis

Nucleic acids from the purified particles were resolved in nine distinct bands by the 10% polyacrylamide gel electrophoresis (Fig. 3). Since the fourth band was thick and broad, the bands were densitometrically analysed. The peak height of the fourth band was about twice that of the other bands, suggesting comigration of two segments. It was separated into two bands by electrophoresis on an agarose–acrylamide composite gel. The virus, therefore, had 10 segments of nucleic acid.

The nature of the nucleic acids was examined by enzyme digestions after electrophoresis in an agarose–acrylamide composite gel. The following nucleic acids were used as standards; dsRNA of rice dwarf virus (RDV), a *Hin*dIII digest of the dsDNA genome of λ phage, and ssRNA of tobacco mosaic virus and 16S and 23S ribosomal RNAs of *Escherichia coli*. The nucleic acids of the virus were resistant to DNase I and RNase A in 2 × SSC (0·3 M-sodium chloride, 0·03 M-sodium citrate, pH 7·0), and were susceptible to RNase A in 0·1 × SSC. The nucleic acids fluoresced green after acridine orange staining. These results were the same as those for RDV and indicate that the genome of NLRV is dsRNA.

Since NLRV had a genome composition which is characteristic for reoviruses, the genome of NLRV was compared with those of four reoviruses transmitted by rice planthoppers and leafhoppers and of a reovirus from an insect. These were (i) rice black-streaked dwarf virus (RBSDV), a fijivirus transmitted by the small brown planthopper *Laodelphax striatellus*, (ii) RRSV, a fijivirus transmitted by the brown planthopper, *N. lugens*, (iii) RDV, a phytoreovirus transmitted by the green rice leafhopper, *Nephotettix cincticeps*, (iv) rice gall dwarf virus (RGDV), a phytoreovirus transmitted by the rice leafhoppers, *Nephotettix nigropictus*, *N. virescens* and *Recilia dorsalis* and (v) cytoplasmic polyhedrosis virus of the silkworm, *Bombyx mori* (BmCPV). RBSDV, RRSV and BmCPV have 10 segments of dsRNAs and RDV and RGDV have 12 segments. The genome composition of NLRV was different from that of the other reoviruses tested (Fig. 3).

The M_r values of the RNA segments of NLRV were estimated by comparing their mobility with those of RDV in 1% agarose gel electrophoresis. Exact M_r values for the segments of RDV were obtained by comparison to the fully sequenced segments, S3 to S10 of RDV (Uyeda *et al.*, 1987, 1989, 1990; Omura *et al.*, 1988, 1989; Fukumoto *et al.*, 1989; Nakashima *et al.*, 1990; Suzuki *et*

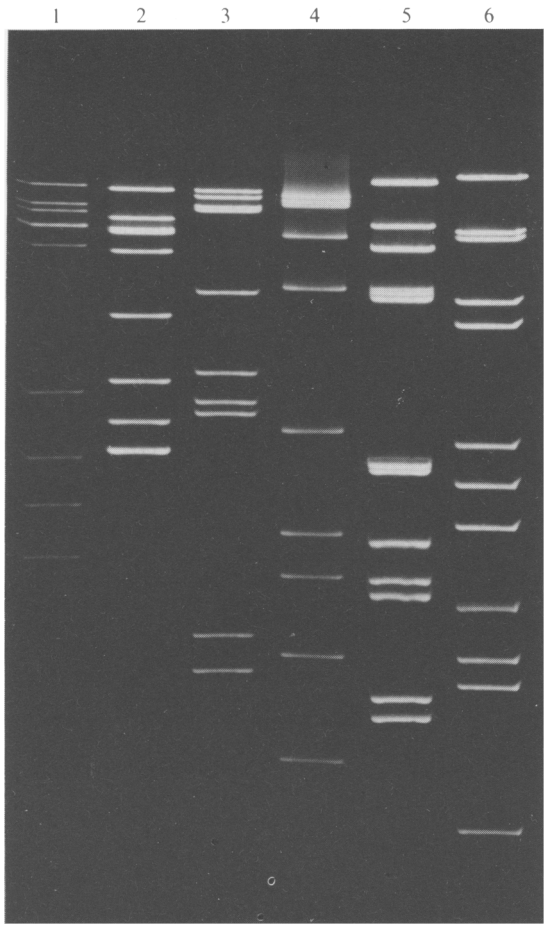

Fig. 3. Electrophoresis of RNA of reoviruses in a 10% polyacrylamide gel stained with ethidium bromide. Lane 1, virus from *N. lugens* (NLRV); lane 2, RBSDV; lane 3, RRSV; lane 4, BmCPV; lane 5, RDV; lane 6, RGDV.

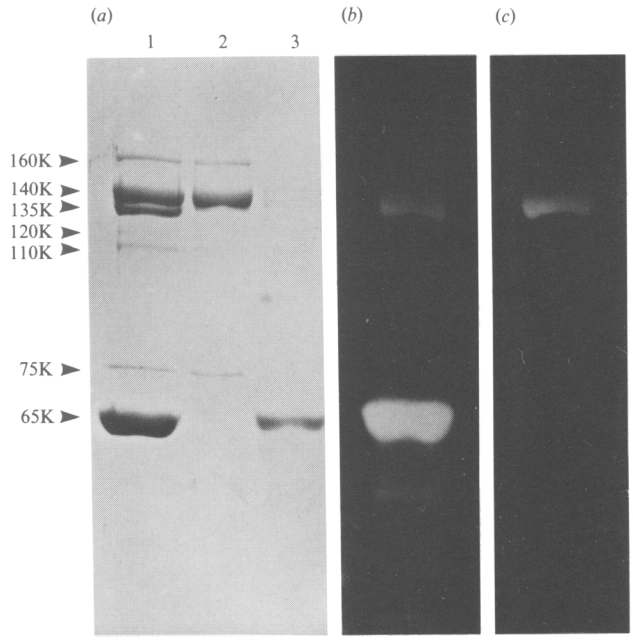

Fig. 4. SDS–polyacrylamide gel (10%) electrophoresis and immunoblotting of virus (NLRV) proteins. (*a*) Proteins from intact particles (lane 1), inner cores (lane 2) and outer shell (lane 3); (*b*) immunoblotting with antiserum to intact particles; (*c*) immunoblotting with antiserum to cores. Proteins were stained with Coomassie blue R250 or blotted onto nitrocellulose paper and stained with a fluorescein isothiocyanate-conjugated second antibody after antiserum treatment.

al., 1990*a*, *b*,). The estimated M_r of the viral genome was $17{\cdot}99 \times 10^6$ and the M_r values of segments S1 to S10 were $2{\cdot}56$, $2{\cdot}31$, $2{\cdot}31$, $2{\cdot}20$, $2{\cdot}17$, $1{\cdot}95$, $1{\cdot}30$, $1{\cdot}18$, $1{\cdot}08$ and $0{\cdot}93 \times 10^6$, respectively.

Protein analysis

Proteins of NLRV were resolved by PAGE into three major components (140K, 135K, and 65K), three intermediate (160K, 110K and 75K) and one minor (120K) (Fig. 4*a*, lane 1). Four of these components (160K, 140K, 110K, and 75K) were recognized in the core particles fraction (Fig. 4*a*, lane 2), and the other three (135K, 120K and 65K) were those removed from the core by the $MgCl_2$ treatment (Fig. 4*a*, lane 3). Although a faint band of the 140K protein was also detected in the supernatant (lane 3), it seemed to be a contaminant from the core protein because it existed in a large amount in the core. The discrepancy of the mobility of 135K proteins between lanes 1 and 3 is thought to be brought about by the different amounts of protein loaded.

Immunoblotting

Immunoblotting showed that antiserum against intact NLRV reacted mainly with the 65K protein (Fig. 4*b*), whereas the antiserum against the core particles reacted mainly with 140K protein (Fig. 4*c*).

Detection of virus antigen

An ELISA was developed to detect NLRV in planthoppers. Although a non-specific reaction was observed in *N. lugens* as pointed out by Hibino & Kimura (1982), the virus antigen was readily detected in the Izumo colony and not detected in the Chikugo colony (Fig. 5).

Vertical transmission of NLRV

Purified NLRV was injected into the virus-free planthoppers of the Chikugo colony to examine vertical transmission of NLRV. The nymphs of the next

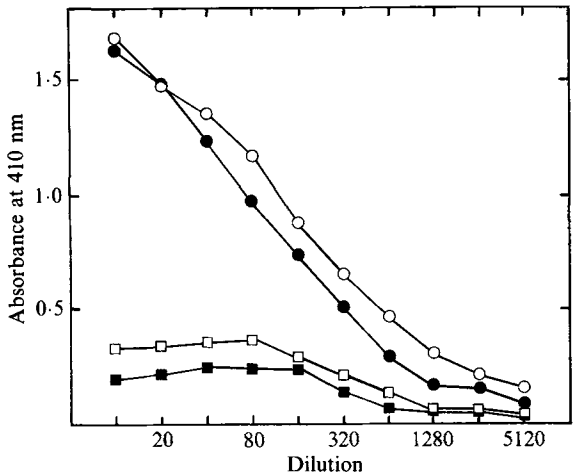

Fig. 5. ELISAs for detecting NLRV in homogenates of (●) planthoppers collected in Izumo in 1987 and (■) planthoppers collected in Chikugo in 1989; open symbols, female; closed symbols, male.

generation of the injected planthoppers gave positive reactions in the ELISA. The virus antigen was detected in the progeny of the virus-injected colony after three generations (data not shown). NLRV was not detected in the rice plants on which the planthoppers had been reared.

Discussion

The polyhedral particle about 65 nm in diameter purified from *N. lugens* (Fig. 2) was proved to be a nucleoprotein (Fig. 3 and Fig. 4). Particle antigens were detected in the progeny of the insect colony injected with purified particles. Hence the particle is a vertically transmitted virus of the planthopper. This virus had dsRNA separable into 10 segments, and appeared in tubules in host cells. These results demonstrate that the virus belongs to the Reoviridae. Several reoviruses have been reported to multiply in leafhoppers and planthoppers (Milne & Lovisolo, 1977; Nault & Ammar, 1989; Shikata, 1989). These are wound tumour virus, RDV, RGDV, RRSV, Fiji disease virus, maize rough dwarf virus, RBSDV, pangola stunt virus, oat sterile dwarf virus, cereal tillering disease virus, maize wallaby ear virus, leafhopper A virus (LAV), reovirus of *Peregrinus maidis* (PMV, PgMV), possibly arrhenatherum blue dwarf virus and lolium enation disease virus.

Among these viruses, RDV, RGDV, RRSV and RBSDV are pathogens of the rice plant and RRSV multiplies in *N. lugens*. NLRV is, however, different from RRSV in the following respects. (i) Symptoms were not observed in host rice plants on which the Izumo colony of *N. lugens* was reared; (ii) NLRV was vertically transmitted to the next generation whereas RRSV was not (Hibino, 1989); (iii) the electrophoretic profile of the genome of NLRV was different from that of RRSV; (iv) no serological cross-reaction was observed between NLRV and RRSV (unpublished data); (v) the RRSV particle has a B-spiked core (Hagiwara *et al.*, 1986) but NLRV showed a typical double shell (Fig. 2*a*).

Two other reoviruses are known to be transmitted vertically in a planthopper and a leafhopper. *P. maidis* harbours a reovirus (PMV, PgMV) naturally (Helord & Munz, 1967; Falk et al., 1988) which has 12 segments of dsRNA (Falk *et al.*, 1988). LAV is found in *Cicadulina bimaculata* and has 10 segments of dsRNA (Boccardo *et al.*, 1980; Ofori & Francki, 1985). Although the host insect of LAV is a leafhopper and is different from that of NLRV, further comparison between NLRV and LAV may be required. An intimate association between auchenorrhynchous vectors and the plant reoviruses is suggested by the fact that these viruses are less harmful to the vector insects than to their plant hosts. Because the reoviruses LAV and PMV infect hoppers, Nault & Ammar (1989) have suggested that plant reoviruses have an insect origin, because they multiply in and are transmitted by insects. The third hopper-infecting reovirus, NLRV, may support the idea of an insect origin for plant reoviruses.

Two reoviruses are also found in *Drosophila*. One is in the cell line of *D. melanogaster*, and has 10 segments of dsRNA (Alatortsev *et al.*, 1981). The other is *Drosophila* S virus (DSV) in *D. simulans*. DSV causes abnormalities of bristles (Louis *et al.*, 1988; Garcia-Vazquez *et al.*, 1989). These viruses, PMV, LAV and DSV, are all hereditary viruses in insects, and both virus-infected and virus-free colonies can be obtained as for NLRV. These viruses are non-pathogenic but seem to be commensals.

Both NLRV and RRSV multiply in *N. lugens*. The vertical transmission of NLRV suggests that this virus has a high affinity with the host insect, which seems to be absent for RRSV. On the other hand, RRSV infects rice plants, in which NLRV does not cause any symptom. Therefore, these viruses may be useful as a model to study the interrelationships between the virus and host plants and between the virus and host insects. Such a comparative study may reveal some important processes in the infection of insect and plant cells and vertical transmission through insects.

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