Multiplication of the Oat Blue Dwarf Virus in the Aster Leafhopper

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ABSTRACT

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Serial passage of the oat blue dwarf virus (OBDV) through eight populations of previously virus-free aster leafhoppers, maintained on OBDV-immune host plants, showed that the leafhoppers transmitted the virus at a dilution of 1×10^{-18} when the original dilution end-point of the inoculum was 1×10^{-5}. Electron microscopic studies of 21 infected insects disclosed crystalline and paracrystalline aggregates of OBDV virion-sized particles in the cytoplasm of cells in the neural lamella of the supraesophageal ganglia and in fat body cells. The virus aggregates were similar to those observed previously in phloem of infected plants. The long minimum incubation period, no transovarial passage of virus, electron microscopic observations of OBDV aggregates in the vector, and the 1×10^{13} increase of virus in the vector during serial passage experiments lead to the conclusion that OBDV multiplies in its insect vector.

The oat blue dwarf virus (OBDV) is an isometric (28- to 30-nm), single-stranded RNA virus (2, 15). In plant hosts, the virus is phloem-restricted and causes hyperplasia of phloem elements in early development (22). The virus has a wide plant host range and is found in low titer in plants (2, 21). The virus is obligately leafhopper-transmitted, and the only recorded vector in North America is the aster leafhopper *Macrosteles fascifrons* (Stål) (1, 3). About 30% of individual insects in wild populations of *M. fascifrons* transmit OBDV, although higher percentages of insects transmit OBDV in populations developed by selective breeding of the vector (9, 19). The virus is not transovarially transmitted in its vector (1, 19). Leafhopper-transmitted pathogenic agents causing similar disease symptoms in oats have been reported in Europe and Africa (8, 10, 16).

Proof that insect-transmitted plant pathogenic viruses multiply in their vectors rests largely on three types of evidence: (i) a relatively long incubation period (measured in days) in the vector between acquisition and the ability to transmit the virus to plants; (ii) serial passage experiments in which a dilution end-point of the virus is established and then greatly exceeded by serial passage and dilution of the virus through successive populations of previously virus-free vectors maintained on immune plant hosts; and (iii) location of intracellular virus aggregates in vector cells by electron microscopy.

In a previous investigation, Banttari and Zeyen (3) used life histories of over 220 adult aster leafhoppers to demonstrate that insects that acquired OBDV by feeding on infected plants required a minimal incubation time of 6 days (20-25 C) before the virus was transmitted to plants. In subsequent research, Chevone and Zeyen *(personal communication),* using 275 age-synchronized leafhoppers, found that the minimum latent period of the virus in males and females was 8 and 5 days, respectively. In addition to the 5-day minimum incubation period, the frequency of daily transmission of insects assayed individually and daily increased slowly but progressively up to 30 days after acquisition feeding, after which the rate of transmission of virus diminished.

If the OBDV – *M. fascifrons* relationship were of a simple circulative nature, the vectors would transmit virus much earlier than 5 days and the transmission frequency should start out high and decline rapidly. In both incubation period and transmission frequency, OBDV differs sharply with a virus-vector combination such as sugarbeet curly top virus (SBCTV) in *Circulifer tenellus* (Baker), which is considered an excellent example of a circulative, nonpropagative, virus-vector relationship (7). Reported here are the results of serial passage and electron microscopic investigations that provide substantial evidence that the OBDV multiplies in its vector.

MATERIALS AND METHODS

The common oat isolate of OBDV (ATCC PV-151) was used throughout. The virus was propagated in oats, *Avena sativa* L. 'Rodney'. Virus-free leafhoppers were maintained on China asters, *Callistephus chinensis* Nees, a plant host immune to OBDV (1, 21). As an added precaution, portions of the virus-free leafhopper population were periodically assayed on oat and flax seedlings *Linum usitatissimum* L. Individual insects were assayed in 26 × 5-cm cylindrical plastic cages set over individual assay plants, after 10-12 days each insect was transferred to a second seedling; thus, each insect had 20-24 days in which to transmit virus to two plants. All transmission studies were made in a greenhouse at 20-25 C and supplemental illumination was used during fall and winter to provide 16 hours of light.

Dilution end-point experiments.—About 200 virus-free leafhoppers in each experiment were allowed to

acquire OBDV by feeding on infected oat plants for 1 week. After acquisition feeding, the insects were placed on the immune host, asters, for an additional week. Twenty to 40 leafhoppers were assayed for OBDV on seedling oats and 100 were ground in a tissue homogenizer in 3 ml of 0.01 M phosphate buffer pH 7.0. The ground insect suspension then was centrifuged at 2,900 *g* for 15 minutes and the supernatant was filtered through cotton. The filtered supernatant then was diluted with buffer to a final concentration of 1:100 (w/v, leafhopper weight in grams to milliliters of buffer). About 250 virus-free leafhoppers were anaesthetized with CO_2 gas and abdominally injected (3) with the following dilutions of the initial leafhopper extract: 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7}. Injected leafhoppers were caged on asters 10 days and then individually assayed twice on seedling oats for two 10- to 12-day periods. Three separate dilution end-point experiments were completed.

Serial passage of the virus through populations of the insect vector.—Inoculum for these experiments was from the same source as that used in dilution end-point experiments. Virus-free leafhoppers (approximately 250) were injected with a 1×10^{-2} dilution of the ground leafhopper inoculum described above. The infected insects were caged on OBDV-immune asters for 14-21 days, after which 20-40 insects were assayed on oats using the same procedures as in the dilution end-point experiments. The remaining insects were ground and used as inoculum, at a 1×10^{-2} dilution, for the next cycle of serial passage to another leafhopper population. Thus, each serial passage resulted in a 1×10^{-2} dilution in the new vector population, except for one dilution of 1×10^{-4} at the end of experiment 3.

Immune-host experiments.—Asters were used as OBDV-immune hosts for leafhopper feeding during serial passage experiments. Although asters previously had been determined to be OBDV-immune (1, 21) we designed an additional experiment to confirm this finding.

During the third serial passage experiment we saved the groups of aster plants used for the fourth-, fifth-, sixth-, and seventh-cycle feedings after the serial-transfer insects were removed. Virus-free leafhoppers plus progeny of the serial-passage insects that had hatched and matured on the aster plants were allowed to feed from 7-14 days on the asters and then the insects were transferred to seedling oats for OBDV assay. All leafhoppers were transferred to at least two sets of oat plants and allowed a minimum 6-day feeding period on each set of seedlings. Fourth-cycle aster plants were assayed for OBDV with 75 insects, fifth-cycle plants with 109 insects, and sixth- and seventh-cycle plants with 195 insects.

Electron microscopy.—Leafhoppers selected for

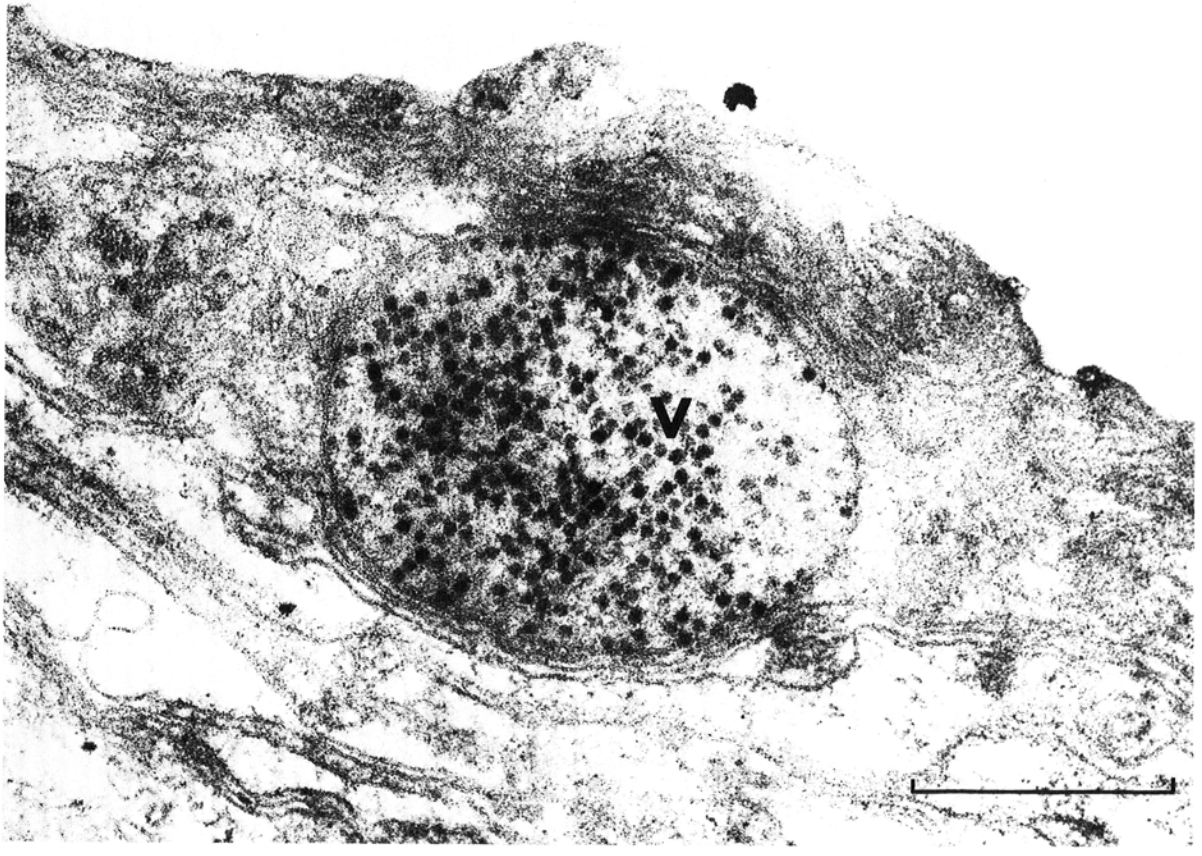

Fig. 1. Membrane-bounded aggregate of oat blue dwarf virus (OBDV) particles in the cytoplasm of cells of the neural lamella surrounding the supraesophageal ganglia of an adult aster leafhopper. V = OBD virions. Bar = 1 μm.

electron microscopic investigations were reared on OBDV-infected oats and assayed on oats to insure that they were OBDV transmitters. Virus-free insects reared on immune hosts were used as controls. Selected insects were anaesthetized with CO_2 gas and fastened dorsally with adhesive to a glass spot plate. The plate containing the insect was flooded with 6% glutaraldehyde (0.01 M phosphate buffer pH 7.0, approximately 5 C) and the insect was dissected.

Tissue from leafhopper brains, salivary glands, malpighian tubules, gut, ovaries, fat bodies, testes, mycetomes, and muscle were fixed for 12-18 hours in buffered 4% glutaraldehyde and postfixed for 1 hour in 2% unbuffered osmium tetroxide. Tissues were dehydrated in acetone, embedded in Luft's medium (12), and sectioned with glass and diamond knives. Sections were stained with saturated uranyl acetate and Venable's lead citrate (20). Twenty-one viruliferous and eight virus-free insects were examined.

RESULTS

Dilution end-point.—To determine the dilution end-point of leafhopper extracts used to inoculate virus-free insects, three experiments were made. In the first, two of 25 insects transmitted virus at a 1×10^{-4} dilution, but no insect transmitted OBDV at greater dilutions. In the second and third experiments respectively, three of 25 and five of 40 insects transmitted virus at a dilution of 1×10^{-5} and there was no virus transmission beyond this latter dilution.

Serial passage of the virus through populations of the vector.—In the first of three serial passage experiments, eight of 25 leafhoppers transmitted OBDV in the fifth population inoculated, resulting in a dilution of the virus from the originally ground insects of 1×10^{-10}. The corresponding dilution end-point experiment ended at a 1×10^{-4} dilution. In the second serial passage experiment two of 25 leafhoppers transmitted the virus in the seventh

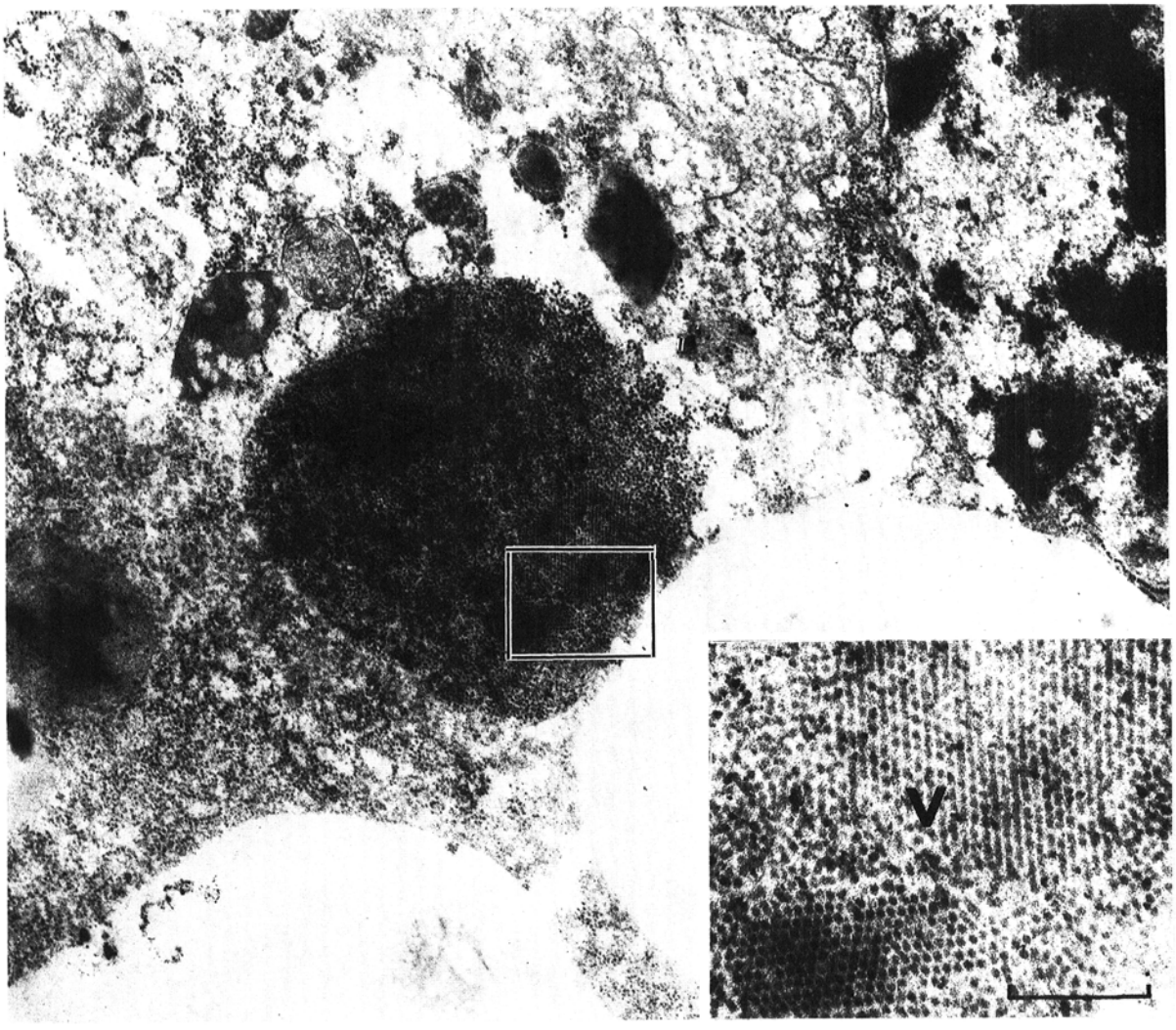

Fig. 2. Crystalline and paracrystalline aggregate, not membrane-bounded, of oat blue dwarf virus (OBDV) particles in the cytoplasm of a cell from a fat body of an adult insect. Inset is an enlargement of the area enclosed by the rectangle. V = OBD virions. Bar = 1 μm.

population of insects for a dilution of 1×10^{-14}. The corresponding dilution end-point of the inoculum obtained from the original ground leafhoppers was 1×10^{-5}. In the third serial passage experiment, 11 of 40 insects transmitted virus in the eighth population for a dilution of 1×10^{-18}. The corresponding dilution end-point of the original inoculum for this serial passage experiment was 1×10^{-5}.

Immune host.—A total of 379 virus-free insects were allowed to feed for 7-21 days on asters used as OBDV-immune host plants. When these 379 insects were transferred at least twice to new seedling oats for OBDV assay, none transmitted the virus. This experiment confirms the earlier experiments of Banttari and Moore (1) and Westdal (21) who originally reported immunity of asters to OBDV.

Electron microscopy.—Extensive examination was made of tissues of 21 OBDV-infected insects and of eight virus-free controls. Membrane-bounded crystalline and paracrystalline aggregates of spherical particles were found in cells of the neural lamella surrounding the supraesophageal ganglia of infected leafhoppers (Fig. 1). The size of the particles and appearance of the aggregates was similar to aggregates of OBDV particles described in phloem of oats (22). Aggregates or recognizable particles were not found in nervous tissue below the cells of the lamella. Large crystalline and paracrystalline inclusions of OBDV-like particles also were found in the cytoplasm of cells found in fat bodies (Fig. 2). The large arrays of particles in fat cells were not membrane-bounded, however, this situation also was previously noted in certain instances in phloem cells of plants (22).

Aggregates of OBDV-like particles were not abundant, nor were they found in every virus-infected insect, even with extensive sectioning of tissues. Crystalline and paracrystalline inclusions of particles resembling OBDV never were found in healthy control insect tissue. Various structures resembling symbiotic microorganisms were noted, especially in the gut and mycetomes, in both healthy and infected insects. Most of these symbiotes were bacteroid in appearance and were similar to those described for *Helochara communis* (6).

DISCUSSION

Three types of evidence commonly are accepted for multiplication of a plant pathogenic virus in an insect vector: (i) a long and consistent incubation period, measured in days, between uptake of the virus and transmission to plants (13); (ii) serial dilution, in which a dilution end-point is established and then greatly exceeded by serial passage of the virus through successive populations of previously virus-free vectors, maintained on immune hosts (4, 18); and (iii) intracellular location of virus particles in vector cells (14, 17). These three lines of evidence when tested experimentally for the OBDV in *M. fascifrons* strongly support the conclusion that OBDV multiplies in its insect vector.

The 6-day minimum incubation period [5 days according to Chevone and Zeyen, *personal communication* and Long and Timian (11)] following acquisition feeding of OBDV, observed in over 475 life histories of individual insects is strong circumstantial evidence for multiplication. The difference between a virus that multiplies in its vector (OBDV) and a virus that is simply circulative (SBCTV) can be graphically illustrated by plotting daily transmissions, using limited acquisition feeding times, against time (Fig. 3). The SBCTV in its leafhopper vector, *Circulifer tenellus* (Baker), is transmitted at a high percent daily transmission on the first day after acquisition feeding ceases and then transmission rapidly decreases as the nonrenewable virus titer drops (7). In the case of OBDV and *M. fascifrons* the virus transmission did not begin until the 6th day and the percent transmission gradually increased to a maximum around 28 days and then gradually decreased. Thus, the long incubation period of OBDV, coupled with the gradual rise in transmission contrasts sharply with SBCTV transmission that begins immediately, at a high rate, and decreases rapidly (Fig. 3).

Serial passage of OBDV through populations of previously virus-free vectors, shows that the dilution end-points were exceeded by 10^6, 10^9, and 10^{13} in three separate investigations. Since the OBDV is not transovarially transmitted (1, 19), and the additional virus was not acquired during feeding on asters, the virus must have multiplied in the successive populations during the serial dilution experiments.

The finding of large concentrations of OBDV-like particles, similar to those described in phloem of infected plants, in the cytoplasm of vector cells (Fig. 1 and 2) can be considered evidence of multiplication, since transport and aggregation of large numbers of particles into cells of tissues not associated with the lining of the insect gut lumen is highly unlikely. Aggregations of particles were observed only in the cytoplasm of cells and nuclei appeared normal. The OBDV virions were not found in abundance in all infected and assayed insects. This may be attributed to age and to the fact that certain insects are less efficient vectors and may have reduced virus titers.

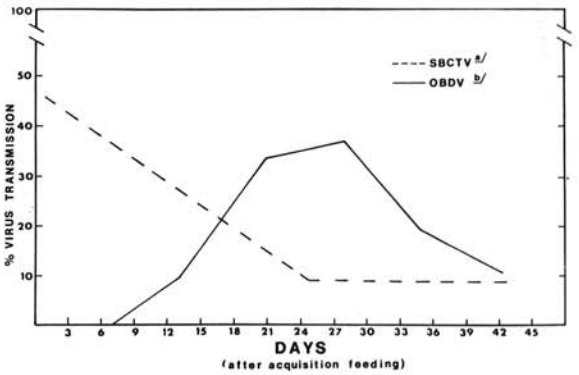

Fig. 3. Comparison of transmission data for the circulative transmission of sugarbeet curly top virus (SBCTV) in *Circulifer tenellus* with the pattern of propagative transmission of OBDV in *Macrosteles fascifrons*. (a) Transmission data for SBCTV (sugarbeet curly top virus) taken from life histories of nine beet leafhoppers fed 0.25-1 day on infected plants, Fig. 4, Freitag 1936 (7). Points determined using a moving average of three successive observations, line fitted to data using linear regression (5). Data averaged due to small sample of insect histories available. (b) Transmission data for OBDV taken from 228 life histories of adult aster leafhoppers fed 1-3 days on infected plants, Banttari and Zeyen, 1970 (3). Points for line determined by means calculated at 7-day intervals.

Inefficiency in virus transmission of leafhopper vectors previously has been noted and apparently is not uncommon (14). Regardless of efficiency of virus transmission, the observations of virions of the wound tumor virus in cells of *Agallia constricta* (Van Duzee) has been considered positive evidence of multiplication (14, 17). The appearance of virus particles in leafhopper tissues not in contact with the absorptive gut lining has been taken as strong evidence for virus multiplication of rice dwarf virus in cells of *Nephottetix cinciteps* (Uhl) and of maize mosaic virus in cells of the planthopper *Peregrinus maidis* (14).

To our knowledge, OBDV is the smallest (approximately 30 nm) single-stranded RNA virus for which evidence of multiplication in both plants and insects has been demonstrated. The OBDV appears to be a specialized phytoarbovirus, being phloem-restricted, inciting hyperplasia of phloeic procambium in plants, and also multiplying in its insect vector. Although *M. fascifrons* is the only known vector of OBDV in North America, similar diseases, as determined by host symptoms and leafhopper transmission, have been reported from Europe (10, 16) and Africa (8). It is highly probable that investigations of certain of these diseases similar to oat blue dwarf may reveal multiplication of other small, isometric plant viruses in Cicadellid vectors.

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