Use of Injected *Macrosiphum euphorbiae* Aphids as Surrogate Vectors for Transfer of Strawberry Crinkle Virus to *Nicotiana* Species

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ABSTRACT

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The polyphagous pink and green potato aphid (*Macrosiphum euphorbiae*) did not acquire strawberry crinkle virus (SCV) from Alpine strawberry (*Fragaria vesca*) by feeding. When infected with SCV by injection, it transmitted the virus to Alpine test seedlings and to *Nicotiana glutinosa* and *N. clevelandii*. Both *Nicotiana* species, when infected, developed symptoms, and rhabdoviruslike particles were found in negatively stained leaf-dip preparations examined in the electron microscope. An extract from symptomatic leaves of *N. glutinosa* was used to inoculate healthy *M. euphorbiae*, and 5% of the injected aphids transmitted virus to Alpine test seedlings. An electron microscopic examination of a negatively stained preparation of a symptomatic flower petal from one of the infected Alpine seedlings contained rhabdoviruslike particles. Furthermore, *Chaetosiphon fragaefolii* reared on this plant subsequently transmitted SCV to other test seedlings. Attempts to mechanically transmit the virus from the two susceptible *Nicotiana* species failed. Comparative data indicated that *M. euphorbiae* was a somewhat less competent vector than *C. fragaefolii* when transmitting SCV to Alpine strawberry test seedlings.

Additional key words: host range

The use of vectors can be crucial in host range studies of vector-dependent prokaryotes and viruses, particularly when grafting or the use of dodder are the only other options available. When suctorial vectors are sufficiently polyphagous to permit efficient acquisition from and inoculation to a variety of host plants, testing procedures are straightforward, but host-plant specificity of the vector can become a limiting factor in experimental work, particularly with acquisition.

Although a relatively low proportion of monophagous *Chaetosiphon* aphids (7) reared on Alpine strawberry (*Fragaria vesca* L. var. *semperflorens* (Duch.) Ser.) plants infected with strawberry crinkle virus (SCV) will transmit virus to test seedlings (5,11,12,16), a high proportion of infective and transmitting individuals can be obtained by injection (15). *Myzus ornatus* Laing is a polyphagous species (7) that will reproduce on strawberry, but apparently, it does not acquire SCV by feeding. However, it also can be inoculated by injection, and although a high proportion of the injected aphids become infected, transmissions to Alpine strawberry test seedlings are rare (14).

Macrosiphum euphorbiae (Thomas) is another polyphagous aphid (7) that will feed and reproduce on strawberry. This report concerns the successful use of injection to inoculate *M. euphorbiae* with SCV and its subsequent transmission to Alpine test seedlings and to *Nicotiana clevelandii* A. Gray and *N. glutinosa* L.

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MATERIALS AND METHODS

Virus source. SCV, originally obtained from a commercial cultivar designated from the group C10 by Frazier (5), has since been maintained in our laboratory by feeding or injecting *Chaetosiphon* aphid species.

The virus used for testing the transmissibility by *M. euphorbiae* to the two *Nicotiana* species originally came from a donor that had acquired SCV by feeding. It had, however, been passed by injection, in both *C. jacobi* H.R.L. and *C. fragaefolii* (Cockerell) several times over a period of 4 yr. Between passages, the source insects were frozen at −65 C. In some instances, the inoculum was pooled from each of the *Chaetosiphon* species. The infected status of all donors was established by prior transmission to Alpine test plants or by finding particles in the electron microscopic examination of a negatively stained preparation of a head dip (15).

Vectors. Two clonal lines of *C. jacobi* and *C. fragaefolii* have been used interchangeably to maintain the SCV source over a period of years. Both species were raised by daily deposition of larvae by mature apterae onto Alpine strawberry seedlings in growth chambers with 13 hr of light at about 19 C and 11 hr of darkness at about 11 C. The *M. euphorbiae* used in this work was initially collected on squash in the field at Berkeley, established as a clonal line, and raised on sowthistle (*Sonchus oleraceus* L.) seedlings in growth chambers with 12 hr of light at about 21 C and 12 hr of darkness at about 15 C. These procedures ensured a regular supply of cohorts of comparable age and size, with a maturation age of about 11–12 days for *C. fragaefolii*, 13–14 days for *C. jacobi*, and 10 days for *M. euphorbiae*.

Host plants. The Alpine strawberry and sowthistle test plants were raised from seed in the greenhouse, transplanted at the three-leaf stage into a sand and peat moss mixture in 5-cm plastic pots, and supplemented periodically with commercial fertilizer. Two experimental *Nicotiana* species, *N. clevelandii* and *N. glutinosa*, were tested for their susceptibility to SCV. These were raised and transplanted as described, and when established and about 3 cm high, they were transplanted to 13-cm clay pots.

Feeding experiments. In a preliminary trial, adult *M. euphorbiae* apterae were allowed to larviposit for 48 hr at 25 C and constant light on a SCV-infected *F. vesca* 'Alpine' seedling previously inoculated by *M. euphorbiae*. One week later, 50 surviving larvae were caged singly on healthy *F. vesca* seedlings for 72 hr at 20 C and constant light and thereafter transferred at 24-hr intervals to fresh Alpine test seedlings for 3 wk.

In a second experiment, 30 2-day-old *M. euphorbiae* larvae were caged on each of two *F. vesca* seedlings at an early stage of infection (8 days postinoculation) with SCV. One source had been inoculated by an injected *C. fragaefolii* and the other by an injected *M. euphorbiae*. The experiment was carried out at 20 C and constant light. The test larvae tended to wander from the source plants, so they were removed after 24 hr and put back onto *Sonchus* seedlings. The 24-hr alternations between SCV-infected Alpine and healthy *Sonchus* seedlings were repeated for two more cycles, giving a total access to the virus source of 72 hr. After two more 24-hr feedings on *Sonchus*, the insects were moved alternately at 24- or 48-hr intervals to Alpine test seedlings and *Sonchus* until death.

Injection. In preliminary tests, the

ability of *M. euphorbiae* aphids to transmit SCV to Alpine strawberry was compared with *C. jacobi* and *C. fragaefolii* after injection. The inoculum was an extract made by grinding the head of a *C. jacobi*, which had acquired SCV-C10 by feeding, in 5 μl of cold distilled water (15). In a second test, the donor aphids were two *C. jacobi* that had been injected in the first test and frozen at −65 C 16 days later. Two months after freezing, the inoculum was prepared by pooling the heads in 10 μl of cold distilled water and injected into *M. euphorbiae*. In a final comparative transmission test, both *C. fragaefolii* and *M. euphorbiae* were injected. The donor aphids were two *C. fragaefolii* that had been frozen at −65 C, 16 days after injection, for 10 mo before use. Again, the inoculum was made by pooling the triturated heads of both aphids in 10 μl of distilled water. The control series of *M. euphorbiae* was injected with distilled water.

Infectivity testing procedure. Injected insects normally were caged in groups of five per Alpine seedling, moved to fresh plants after 48–72 hr, and then placed singly on test plants 5 or 6 days postinoculation. This gave us a record of the transmission rate before the aphids were recombined into groups and caged on the *Nicotiana* species. The groups of test insects were transferred daily to fresh *Nicotiana* test plants that were about 5–8 cm high. Insect-free *Nicotiana* plants of comparable size were caged at irregular intervals to serve as controls. All inoculation access periods were done in a growth chamber set for constant light and 18.5 ± 0.7 C. In some early experiments, the *M. euphorbiae* were caged on sowthistle seedlings (their maintenance host plant) after injection in an attempt to lessen the added trauma of feeding on Alpine strawberry, a transitional test plant. Sowthistle and Alpine seedlings were also alternated with the tobacco plants to aid survival when in the initial tests *M. euphorbiae* tended to reject tobacco and wander in the cage. This procedure, however, was discontinued in later experiments when tobacco proved to be an adequate host plant.

In the experiment comparing transmission by *C. fragaefolii* and *M. euphorbiae* to Alpine strawberry test seedlings, the test access periods were at 18.5 ± 0.7 C and constant light of 8,600–11,000 lux and were about 24 hr long. Groups of five aphids per test plant were used during the first two test access periods. Thereafter, the aphids were individually tested by moving them daily to fresh test plants until death.

Mechanical inoculation. In the initial trial, 2.6 g of leaf tissue from a symptomatic *N. glutinosa* (after aphid inoculation with SCV-C10) was ground, using a mortar and pestle, in 27 ml of cold 0.01 M potassium phosphate buffer, pH 7.0, squeezed through two layers of Miracloth, and rubbed on a healthy *N. glutinosa* test plant dusted with Carborundum. At the same time, 0.6 g of symptomatic leaves of *N. clevelandii* with a similar SCV-C10 history was ground in 6.5 ml of the same buffer and mechanically inoculated to *N. clevelandii*. The leaves were rinsed with water after inoculation.

In further tests, attempts were made to inoculate the two *Nicotiana* species using the following extraction buffers: 0.1 M Tris-HCl, pH 8.4, 0.01 M Mg acetate, 0.04 M Na_2SO_3, and 0.001 M $MnCl_2$ (6); 0.04 M Na_2SO_3 (2); and 0.05 M potassium phosphate, pH 7.0, and 0.1% 2-mercaptoethanol (3). In all cases, 1 g of diseased tissue, from either *N. clevelandii* or *N. glutinosa*, was ground on ice in 10 ml of buffer containing 0.1% Celite.

RESULTS

Acquisition by feeding. In the preliminary feeding experiment, none of the 50 *M. euphorbiae* larvae allowed a 7- to 8-day access period on an infected *F. vesca* source plant transmitted SCV to Alpine test seedlings during the 3-wk postacquisition period. In a second feeding experiment, 30 *M. euphorbiae* were given three 24-hr access feedings (72 hr of total acquisition time) on an SCV-infected *F. vesca* Alpine seedling previously inoculated by *M. euphorbiae*, and another 30 were fed on one inoculated by *C. fragaefolii*. Again, none

Table 1. Life table and transmission statistics of *Chaetosiphon fragaefolii* and *Macrosiphum euphorbiae* infected by injection with strawberry crinkle virus (SCV) or water and tested on Alpine strawberry[u]

Estimated parameter	Infected		Control
	C. fragaefolii	*M. euphorbiae*	(*M. euphorbiae*)
Sample size			
Injected	25	45	21
Tested[v]	21	37	18
Infected[w]	20	28	0
Longevity (days)	40.6 a ± 8.7	29.6 b ± 10.4	42.1 a ± 13.4
Net reproductive rate	37.8 a ± 10.3	25.2 b ± 11.0	31.7 ab ± 11.9
Larvae/female/day	1.14	1.10	0.89
Cohort generation time (days)	21.8	16.8	18.3
Capacity for increase	0.17	0.19	0.19
Intrinsic rate of increase (R_m)	0.2070	0.2273	0.2289
Generation time (days)	17.5	14.2	15.1
Finite population increase/day	1.23	1.26	1.26
Doubling time (days)	2.46	2.51	2.51
Injection efficiency	0.95	0.76	
Net transmission rate	10.65 a ± 2.94	8.14 b ± 4.35	
Median latent period (days)[x]	6.6	6.9	
Time of av. transmission (days)	13.0	14.0	
Transmission period (days)	13.8 a ± 5.4	10.5 a ± 8.1	
Retention period (days)	20.8 a ± 5.5	17.6 a ± 8.4	
Retention index[y]	0.62	0.80	
Efficiency index[z]	0.77	0.77	

[u] Infected aphids were obtained by injecting each with an estimated 0.1–0.02 μl of inoculum prepared by triturating the head of a previously SCV-infected donor aphid in 5 μl of distilled water. A group of *M. euphorbiae* injected with a similar amount of distilled water was used as controls. After two initial transfers in groups of five, all aphids were individually transferred at daily intervals until death to Alpine strawberry test seedlings. All inoculation test access periods were done at 20 C and constant light of 8,600–11,000 lux. Where appropriate, the values are the mean ± standard deviation. Means on the same line followed by the same letter lacked significant evidence ($P = 0.05$, t test) of being different. For further details on the calculation of the various life-table statistics and their adaptation to virus transmission see Sylvester and Richardson (J. Econ. Entomol. 59:255-261).

[v] Aphids not tested included those that died within 96 hr of injection, were injured, or were lost prematurely.

[w] The criterion for infection was transmission.

[x] Estimated using linear regression on log-probit-transformed data.

[y] Ratio of the retention period to the postacquisition survival period.

[z] Probability of daily transmission during the transmission period.

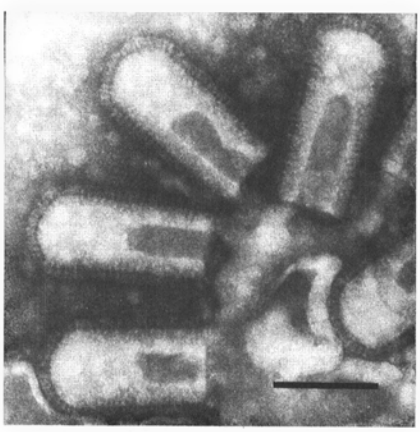

Fig. 1. Bullet-shaped virions in a negatively stained preparation from the head of a *Macrosiphum euphorbiae* injected with strawberry crinkle virus. Scale bar = 100 nm.

transmitted SCV to Alpine test seedlings when tested by daily transfers until death, up to 48 days later.

Injection. *Transmission to F. vesca.* When *M. euphorbiae* was injected with an extract from a *C. jacobi* donor that had acquired SCV by feeding on an Alpine strawberry, seven of nine (78%) transmitted to Alpine test plants compared with 19 of 20 (95%) *C. jacobi* and 11 of 14 (79%) *C. fragaefolii* that were injected with the same inoculum. The expected numbers of nontransmissions were too small to permit a valid chi-square test. In a second trial, 16 of 20 (80%) *M. euphorbiae* transmitted SCV when injected with inoculum made from the pooled heads of two *C. jacobi* from the previous experiment. The presence of virus particles was ascertained in the inoculum before injection and in a head-dip preparation from injected *M. euphorbiae* (Fig. 1).

The life table and transmission data, obtained from a test in which infected *C. fragaefolii* and *M. euphorbiae* were compared, are summarized in Table 1. SCV-injected *C. fragaefolii* tended to live longer, produce more offspring, and have a longer generation time and a lower intrinsic rate of increase than *M. euphorbiae*. The latent periods were similar, and although *C. fragaefolii* tended to inoculate more plants over a somewhat longer period of time than did *M. euphorbiae*, the efficiency index, i.e., the ratio of the number of plants inoculated to the length of the transmission period, was similar for the two species.

M. euphorbiae injected with SCV did not live as long or produce as many offspring as did comparative individuals injected with water. Although the generation time for the infected aphids was somewhat less than for uninfected controls, the estimated time needed for the population to double (2.5 days) was the same for SCV-injected or water-injected aphid samples.

Transmission to Nicotiana species. Seven and 9 days after injection with an extract from an infected *C. fragaefolii*, nine of 21 (43%) and 12 of 21 (57%) *M. euphorbiae*, respectively, transmitted SCV to Alpine strawberry when tested singly. When these insects were pooled into groups of five to seven insects and caged on *N. glutinosa* for a 24-hr test period, two of four transmitted SCV. Using the same inoculum, 13 of 27 (48%) and 18 of 26 (69%) injected *M. euphorbiae* transmitted SCV to *F. vesca* Alpine when tested singly and two of four groups transmitted it to *N. clevelandii.*

Symptomatology. *N. clevelandii.* Symptoms are initiated on the young developing leaves by a slight edge ruffling and development of a coarse light and dark green mosaic. When affected leaves are viewed with transmitted light, the older secondary veins show a discontinuous clearing accompanied by a diffusing chlorosis near the affected areas. In subsequently developing leaves, marginal distortion can occur, resulting in an asymmetrical curving of the leaf. There is some reduction in leaf size, and the leaf shape becomes more lanceolate than in uninfected plants. The internodal growth is reduced, resulting in a severe stunting (Fig. 2) or a rosette appearance of the plant. In some infections of older plants, not all of the stems are equally affected, and some may remain asymptomatic. On symptomatic stems, flowering is reduced, and the flowers tend to become clustered at the tip of the stem because of a decrease in internodal spacing. Many flowers fail to mature or to form seeds.

N. glutinosa. Symptoms begin with chlorosis of the new growth followed by a discontinuous veinclearing and chlorotic spotting. This pattern continues on the newly emerging leaves. As affected leaves mature, there is a gradual transition of the yellow spotting into necrotic lesions surrounded by irregular chlorotic halos. Some of the necrotic lesions may slowly continue to enlarge and coalesce, resulting in considerable leaf necrosis of fully matured or senescing leaves (Fig. 3). Elongation of the internodes is reduced (Fig. 4). Although the flowers appear normal, they can be reduced in number and borne on somewhat shorter flower stalks compared with uninoculated control plants.

Plant extract injection. To confirm that the bacilliform particles observed in the negatively stained preparations from *N. glutinosa* (Fig. 5) were SCV, an extract was prepared from a symptomatic *N. glutinosa* plant by grinding a leaf fragment in cold distilled water and immediately injecting it into *C. fragaefolii* and *M. euphorbiae*. These injected insects were tested for infectivity by transferring them to Alpine seedlings every 24 hr for 3 wk. None of 29 tested *C. fragaefolii* and two of 39 *M. euphorbiae* transmitted SCV. The low rate of successful inoculation of the aphid species suggests that the inoculum was a poor source of virus.

Fig. 2. (Left) Healthy and (right) diseased *Nicotiana clevelandii*. The diseased plant was inoculated 73 days previously by *Macrosiphum euphorbiae* injected with strawberry crinkle virus.

Fig. 3. *Nicotiana glutinosa* leaves infected with strawberry crinkle virus showing a pattern of extensive necrotic lesions. The plant was inoculated 85 days previously by injected *Macrosiphum euphorbiae*.

Fig. 4. (Left) Healthy and (right) diseased *Nicotiana glutinosa*. The diseased plant was inoculated 85 days previously by *Macrosiphum euphorbiae* injected with strawberry crinkle virus.

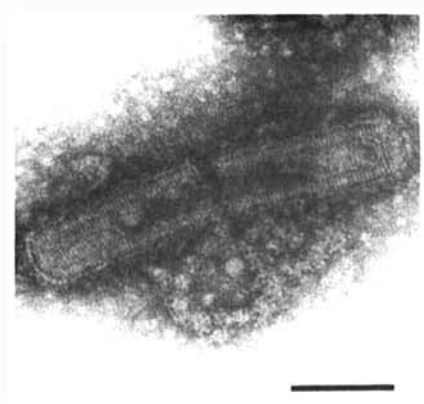

Fig. 5. Disrupted bacilliform particle in a negatively stained preparation from symptomatic *Nicotiana glutinosa* infected with strawberry crinkle virus. Scale bar = 100 nm.

The two resultant symptomatic strawberry plants inoculated by injected *M. euphorbiae* were then used as sources for acquisition feeding by *C. fragaefolii*. A negatively stained preparation of a petal from one of the Alpine source plants proved to have bacilliform and bullet-shaped particles.

SCV recovery by feeding. Of the 60 and 30 *C. fragaefolii* larvae deposited on the two SCV-C10 Alpine source plants that had been inoculated by *N. glutinosa*-extract-injected *M. euphorbiae*, only one acquired SCV during an 11-day acquisition period and transmitted SCV to three consecutive test plants when tested singly.

DISCUSSION

Propagative aphidborne plant rhabdoviruses are quite vector-specific, and most reported vectors are either oligophagous or monophagous (4). In spite of this, it has been possible to inoculate plants not considered to be hosts of the vector. This is true for lettuce necrotic yellows virus, where the monophagous vector *Hyperomyzus lactucae* (L.) could be used to inoculate *N. glutinosa* (9), and for coriander feathery red-vein virus, where both *N. glutinosa* and *N. clevelandii* were infected by the oligophagous aphid vector *Hyadaphis foeniculi* (Pass.) (8). In both of these cases, further transmission among the *Nicotiana* species could be done by mechanical inoculation.

At least two other aphidborne plant rhabdoviruses, viz., sowthistle yellow vein virus (SYVV) and SCV, have not been reported to be mechanically transmissible (10,13), and the major vectors, *Hyperomyzus lactucae* and aphid species in the genus *Chaetosiphon*, respectively, are considered monophagous (7). Host range studies of both of these viruses might be aided considerably if an efficient vector could be found among a suitable polyphagous aphid species. Although *M. euphorbiae* fed on sowthistle will acquire SYVV, it is a very inefficient vector (1) and has not been used in host range tests. In the case of SCV, at least two polyphagous species of aphids, viz., *Myzus ornatus* and *Macrosiphum euphorbiae*, will breed on strawberry, but apparently, neither will acquire SCV by feeding. Although both species can be infected by injection, only *M. euphorbiae* has proven to be a competent vector. Perhaps, the demonstrated use of this latter species as a surrogate vector to transmit SCV to at least two *Nicotiana* species will encourage additional work on the host range and characterization of this important strawberry virus.

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